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(Name and address of applicant, and in case of body corporate-place of incorporation)

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(באנגלית) (English)

AUTOMATION COMPATIBLE DEVICES FOR SCANNING ELECTRON MICROSCOPY IMAGING OF SAMPLES IN A WET ENVIRONMENT

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AUTOMATION COMPATIBLE DEVICES FOR SCANNING ELECTRON MICROSCOPY IMAGING OF SAMPLES IN A WET ENVIRONMENT

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QMX/008 IL

AUTOMATION COMPATIBLE DEVICES FOR SCANNING ELECTRON MICROSCOPY IMAGING OF SAMPLES IN A WET ENVIRONMENT USING

FIELD OF THE INVENTION

The present invention relates to automation compatible devices for the examination of samples in a non-vacuum and a wet environment using a scanning electron microscope, and particularly but not exclusively to the use of such an apparatus for inspection of cells in a physiological environment, or to determine intermolecular interactions.

BACKGROUND OF THE INVENTION

Optical microscopy is limited, by the wavelength of light, to resolutions in the range of a hundred, and usually several hundreds, of nanometer. Scanning electron microscopes (SEMs) do not have this limitation and are able to attain a considerably higher resolution, in the range of a few nanometers.

One of the disadvantages of SEMs is that samples have to be maintained in vacuum, precluding the study of in-vivo processes or the study of wet materials. Furthermore, electrically insulating samples composed of organic materials require coating to avoid charge accumulation.

As early as 1960 a thesis by Thornley (University of Cambridge, 1960) disclosed unsuccessful attempts to maintain a sample intended for electron microscopy in an atmosphere of water vapor. A membrane is used to seal a chamber from the vacuum of the electron beam and the chamber itself has an inlet from a source of water vapor.

Attempts to use Electron Microscopy for living specimens go back as far as 1970. A paper by Swift and Brown (J. Phys. E: Sci. Instrum. **3**, 924, 1970) disclosed the use of transmission electron microscopy (TEM) for examination of specimens at atmospheric

pressure, for example in water. A cell having an aperture sealed with a collodion-carbon film is used to mount a sample. An electron beam passes through the aperture to strike the sample, and electrons not stopped by the sample continue to a scintillator where photons are produced. At atmospheric pressure the results were found to be "rather noisy" although a resolution of 0.1μm was claimed.

US Patent 4,071,766 describes an attempt to use electron microscopy to see material in a non-vacuum environment, and refers to the inspection of living objects as "an ever-recurring problem". US Patent No. 4,720,633 describes a further attempt to use electron microscopy to see material in a non-vacuum environment. In both of these patents the electron beam travels through an aperture to a wet specimen. Neither of these attempts succeeds, however, in effectively viewing wet objects. The contents of both of these documents are hereby incorporated by reference.

A commercial product which attempts to solve the above problem is an Environmental Scanning Electron Microscope (ESEM), commercially available from Philips Electron Optics of Eindhoven, The Netherlands, which maintains a vacuum gradient along the electron beam path. However, the ESEM requires working with a sample at a critical point of water-vapor equilibrium, and requires cooling of the sample to around 4°C. Inspection of specimens at pressures of up to 5 Torr is said to be possible. Further information on this product and how it works can be found in US Patents 5,250,808, 5,362,964, and 5,412,211, the contents of which are hereby incorporated by reference.

A common method of achieving high resolution inspection of organic matter is Transmission Electron Microscopy (TEM). TEM requires specially prepared specimens having typical thicknesses in the range of 50nm. A very high voltage is applied to create a parallel beam that passes through the sample. US Patent 5,406,087, the contents of which

are hereby incorporated by reference, discloses a specimen holding device for use with a TEM. A specimen is sealed between two films that are able to transmit an electron beam. The interior of the device is filled with moisture and may be placed within the vacuum environment of the TEM. A very high energy beam travels through the specimen and surrounding fluid leading to a poor image contrast or signal to noise ratio, as well as considerable damage to the sample.

The information made available by EM is usually unavailable by other techniques (reviewed in Griffiths *Trends in Cell Biology*, 11:*4*:153-154, 2001). The main reason for the prevalent underutilization of EM is the complexity of sample preparation, that is not only labor intensive and time consuming, but also raises concerns regarding the biological relevance of the results. The ability to carry out EM in an aqueous environment would obviate these problematic sample preparation steps.

A co-pending International patent application by one of the present inventors, entitled "Device and method for the examination of samples in a non-vacuum environment using a scanning electron microscope" (PCT/IL10/01108), the content of which is hereby incorporated by reference, discloses a non-vacuum SEM device that allows imaging cells with SEM in a wet environment. This is accomplished by the use of a thin membrane, also termed hereinafter a partition membrane, that is transparent for electrons but is strong enough to withstand the pressure difference between the inside of the chamber and the vacuum required for the electron beam.

The current invention describes further improvements to the previous invention (described in PCT/IL01/01108), designed to render the chamber automation-compatible, and to further enhance its utility for imaging biological samples in physiological conditions.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an automation-compatible chamber, adapted for use with a scanning electron microscope, that enables electron microscopy of single and multiple samples in a wet or fluid environment.

It is another object of the present invention to provide an automation-compatible sample chamber adapted for use with a scanning electron microscopy, that enables electron microscopy of single or multiple samples in a wet or fluid environment and allows a reduced pressure inside the chamber.

It is another object of the present invention to provide an automation-compatible chamber, adapted for use with a scanning electron microscope, that enables electron microscopy of single and multiple samples in a wet or fluid environment, useful for inspection of living cells in physiologically relevant media, as well as for detection of interactions of cells with molecules of interest.

It is another object of the present invention to provide an automation-compatible chamber, adapted for use with a scanning electron microscope that enables electron microscopy of single and multiple samples in a wet or fluid environment, useful for detection of intermolecular interactions.

In general, the sample chamber is located in a vacuum whilst enclosing a sample or samples within a fluid, or at substantially atmospheric pressure, or both.

In accordance with this general aspect of the present invention there is provided an automation-compatible chamber suitable for use with a scanning electron microscope, said chamber comprising at least one aperture, which aperture is sealed with a membrane, said membrane being adapted to withstand a vacuum, and being transparent to electrons and wherein said chamber is isolated from said vacuum.

According to one aspect of the present invention the sample chamber assembly is not

completely filled with fluid, but rather remains partially filled with a compressible gas, thereby facilitating the preparation and handling of the samples. According to a second aspect the sample chamber assembly comprises at least one distensible or elastic element that permits the internal volume of the chamber to vary, thereby achieving a reduced pressure inside the chamber.

According to another aspect of the present invention the sample chamber is open to the vacuum environment, yet the sample remains in a wet environment, said chamber allowing pressure reduction within the chamber to the order of the liquid vapor pressure inside the chamber.

According to another aspect the sample to be imaged is attached to a separate substrate other than the partition membrane that seals the chamber, enabling viewing the surface of the sample that is accessible to the fluid within the sample chamber.

According to yet further aspects of the present invention devices enabling preparation in parallel of multiple samples, and their handling prior to or during examination are disclosed.

In accordance with a first feature the sample chamber is enclosed within a housing said housing having a hollow compartment filled with compressible gas.

In accordance with one particular embodiment of the present invention an automation-compatible chamber comprises:

(a) a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane;

(b) the partition membrane sealing the top of the cavity disc having an outer surface and an inner surface, said inner surface facing the cavity of said cavity disc, said partition membrane being transparent to electrons;

(c) optionally, a grid affixed to the outer surface of the partition membrane;

(d) a frame element for holding the cavity disc, the frame element having an aperture extending at least over the width of the cavity;

(e) a housing surrounding the framed cavity disc having attachment means for attaching the frame to the housing;

(f) said housing enclosing a hollow space containing a compressible gas.

The fact that the sample chamber is housed within a housing having a space filled with a compressible gas, considerably simplifies the procedures of readying the sample chamber for examination in the SEM. This chamber is therefore compatible to the automation of the imaging of samples in a wet environment by scanning electron microscopy.

Optionally and advantageously, a grid may be affixed to the outer surface of the partition membrane, as a physical support reducing the risk of rupture of the partition membrane.

In accordance another aspect of the present invention, it is possible to achieve reduced pressure specifically upon the partition membrane, of the automation-compatible sample chamber by replacing one of said chamber's walls by an elastic material or distensible film. In this aspect the automation-compatible chamber of the invention has a varying internal volume.

According to a particular embodiment of this aspect of the present invention, the automation-compatible elastic chamber comprises:

(a) a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane;

(b) the partition membrane sealing the top of the cavity disc having an outer surface and an inner surface, said inner surface facing the cavity of said cavity disc, said partition membrane being transparent to electrons;

(c) optionally, a grid affixed to the outer surface of the partition membrane;

(d) a frame element for holding the cavity disc, the frame element having an aperture extending at least over the width of the cavity;

(e) a housing having an upper part and a lower part wherein the upper part of the housing extends from the frame on one end to an elastic film at the other end;

(f) a support element placed below the housing and supporting it, having a top surface and a bottom surface surrounding a cavity in the support element through which the elastic film can be stretched; wherein the top of the support element extends to the housing and is covered with the elastic film, the bottom of the support element extends to a plug;

(g) the plug placed at the bottom of the support element defines the maximum extent to which the elastic film can be stretched;

(h) attachment means for attaching the frame to the housing and for attaching the housing to the support element.

Preferably, the elastic or distensible film used for the automation-compatible elastic chamber is selected from materials that are elastic enough to allow a considerable change in the interior volume of the chamber on one hand yet are able to withstand the pressure difference between the interior of the chamber and the surrounding vacuum without rupture or leakage. More preferably, the materials required for a suitable elastic film may be selected from the group comprising: elastomers, elastomers based on polystyrene/elastomer block copolymers, S-B-S, S-EB-S, S-I-S, Parafilm®.

A further element provided for use in conjunction with the automated compatible

elastic chamber of the invention is a stretching tool, wherein the elastic film is pressed through a cavity at the lower part of the housing by said stretching tool. This maximizes the expansion volume of the chamber.

The automation-compatible elastic chamber allows low pressure on the partition membrane and hence reduces the possibility of the partition membrane rupturing even further and allows use of a thinner partition membranes than in the previous chamber designs. Accordingly, the automation-compatible elastic chamber facilitates higher imaging resolution than the resolution achieved with the automation-compatible chamber since with the thinner partition membrane fewer electrons are scattered by the partition membrane.

According to another aspect of the present invention, the automation compatible chamber is an oil chamber wherein a layer of low vapor pressure liquid separates the vacuum environment of the electron microscope from the sample, which is thus maintained in a wet environment.

According to one embodiment of this aspect of the invention the automation-compatible oil chamber comprises:

(a) a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane;

(b) a partition membrane sealing the top of the cavity disc having an outer surface and an inner surface, said inner surface facing the cavity of said cavity disc, said partition membrane being transparent to electrons;

(c) optionally, a grid affixed to the outer surface of the partition membrane;

(d) a frame element for holding the top surface of the cavity disc, the frame element having an aperture extending at least over the width of the cavity;

(e) a layer of separation liquid applied over the sample.

Preferably, the liquid used as a separation layer in the automation-compatible oil chamber should have of the following properties:

(a) low vapor pressure;

(b) immiscibility with an aqueous medium;

(c) high surface tension;

(d) specific gravity lower than that of an aqueous medium.

Preferably, the separation layer used in the automation-compatible oil chamber may be selected from the group comprising: low vapor pressure oils, paraffin oil, TKO-w/7 oil (Kurt J. Lesker Co., Pittsburgh PA, USA), N-methyl pyrrolidone, gamma-butyrolactone.

Preferably, the automation-compatible oil chamber allows low pressure on the partition membrane and hence reduces the possibility of the partition membrane rupturing even further and allows use of a thinner partition membranes than in the other chamber designs. Accordingly, the automation-compatible oil chamber facilitates higher imaging resolution than the resolution achieved with the automation-compatible chamber and the automation-compatible elastic chamber since with the thinner partition membrane fewer electrons are scattered by the partition membrane.

According to yet another aspect of the present invention, the automation-compatible chamber is an inverted chamber designed to enable examination of the surface of a sample that is accessible to the fluid medium in which the sample is placed within said chamber.

According to a particular embodiment of this aspect of the invention the inverted chamber comprises:

(a) a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane;

(b) the partition membrane sealing the top of the cavity disc having an outer surface and an inner surface, said inner surface facing the cavity of said cavity disc, said partition membrane being transparent to electrons;

(c) optionally, a grid glued to the outer surface of the partition membrane;

(d) a frame element for holding the top surface of the cavity disc, the frame element having an aperture extending at least over the width of the cavity;

(e) a substrate having an upper side and a lower side, wherein the upper side faces the partition membrane and a sample is situated on said upper side of said substrate;

(f) means for positioning the substrate in close proximity to the partition membrane;

(g) a housing element enclosing the framed cavity disc and positioning means;

(h) attachment means for attaching the frame to the housing.

In a preferred aspect of the present invention, the substrate on which the sample is situated for use in the automation-compatible inverted chamber includes the following features:

(a) the substrate is located close to the partition membrane and is suitable to the shape of the partition membrane when positioned inside the microscope chamber;

(b) the substrate must not break or disrupt the partition membrane, and therefore should be smooth, having no sharp edges;

(c) optionally, the substrate may be elastic.

For all of the chambers according to the present invention, the properties required of the partition membrane, which constitutes one of the required elements in the chambers of the present invention, include the ability to withstand a pressure differential of approximately one atmosphere between the vacuum of the electron microscope and the

environment contained within the chamber sealed by said membrane, while it is thin enough for energetic electrons to pass through and interact with the sample provided within the chamber. Any material with these attributes may be suitable for use in accordance with the principles of the present invention. According to currently preferred embodiments, the partition membrane is made of a material selected from the group comprising: polyimide, polyamide, polyamide-imide, polyethylene, polypyrrole and additional conducting polymers, parlodion, collodion, Kapton, FormVar, Vinylec, ButVar, Pioloform, silicon dioxide, silicon monoxide, and carbon.

Preferably, the sample to be examined using the chambers of the invention will be positioned on the inner surface of the partition membrane or on a separate substrate in close proximity to the partition membrane. The sample is any specimen that is to be examined under the electron microscope. The chamber of the present invention enables examination of viable cells. Alternatively and preferably cells may be cultured and/or labeled in the cavity of the cavity disc.

Preferably, the cavity disc for use in the chambers of the present invention is cylindrical in shape, having a cavity that is also cylindrical in shape. Additionally and preferably, the cavity disc is made of any plastic that maintains its shape and texture in the temperature range and pressure range which exist in the chamber during sample preparation and during sample imaging. According to a currently most preferred embodiment of the invention, and by way of non-limiting example, the cavity disc is made of Perspex.

Preferably, the attachment means used to attach the housing to the frame include, without limitation, at least one of the following elements: screws, nuts, clips. The attachment means may further comprise sealing means including by way of non-limiting example O-rings, or any appropriate sealant.

According to additional aspects of the invention, the chambers of the invention are held either singly or in arrays of multiple chambers, on a sample holder adapted to the dimensions of the chambers. Advantageously, the sample holder may hold multiple sample chambers. More advantageously, the sample holder is used in conjunction with automation means enabling automated exchange of samples during the examination procedure.

A further element provided for use in conjunction with the chambers of the invention is an ejector, wherein said ejector is placed over the cover of the sample holder and allows its removal. The ejector element is particularly advantageous for use with multiple sample chamber holders.

Preferably, prior to imaging the chambers are placed inside a vacuum chamber under a pressure lower than 10^{-2} mbar, to verify the ability of the chamber to withstand exposure to a vacuum.

Preferably, chambers of the invention are adapted to hold water or any aqueous medium, including but not limited to cell culture medium.

According to yet another aspect of the present invention the samples can be handled inside the multiple sample holder in a fashion that enables liquids to be inserted into the disc cavity and to be drained therefrom. By way of non-limiting example liquids may be inserted with pipettes, whereas they may be drained via a multi-drain connected to a pump, where the multi-drain is designed so that liquids can be removed without damaging the partition membrane.

.In another aspect of the present invention any of the automation-compatible chambers may be designed for repeated usage or may be disposable.

Preferably, the wet sample attached within the devices of the invention for SEM or ESEM viewing, comprises a pharmaceutical composition.

Preferably, said wet sample further comprises a living cell with which the

pharmaceutical composition is interacting.

The chambers and samples of the invention are useful to detect interactions between cells and molecules or for determining intermolecular interactions in solution.

BRIEF DESCRIPTION OF THE DRAWINGS

For a better understanding of the invention and to show how the same may be carried out, reference is now made, purely by way of example, to the accompanying drawings, in which:

FIG. 1a is a generalized cross-sectional diagram of the automation-compatible chamber.

FIG. 1b-c is a picture of an opened (b) and a closed (c) automation-compatible chamber.

FIG. 2a is a generalized cross-sectional diagram of a cavity disc of an automation-compatible chamber.

FIG. 2b is a picture of a cavity disc of an automation-compatible chamber.

FIG. 3 is a generalized cross-sectional diagram of a cavity disc frame of an automation-compatible chamber.

FIG. 4 is a generalized cross-sectional diagram of a housing of an automation-compatible chamber.

FIG. 5 is a generalized cross-sectional diagram of a base of a multi-sample holder.

FIG. 6a is a generalized cross-sectional diagram of a cover of a multi-sample holder.

FIG. 6b-c is a picture of an opened (b) and closed (c) multi-sample holder.

FIG. 7a is a generalized cross-sectional diagram of a multi drain for a multi-sample holder.

FIG. 7b-c is a picture of a multi-drain and a multi-pipette for a multi-sample holder.

FIG. 8 is a generalized cross-sectional diagram of an ejector for a multi-sample holder.

FIG. 9a is a generalized cross-sectional diagram of an automation-compatible elastic chamber.

FIG. 9b shows a SEM image acquired using the automation-compatible elastic chamber of gold labeled HeLa cells with transiently expressing the alpha subunits of IL-2 receptor. The field shows both transfected and non-transfected cells, demonstrating the exquisite signal to noise ratio.

FIG. 9c-h shows a series of images of HeLa cells gold labeled using an anti EGF receptor antibody at different magnifications.

FIG. 9i shows an image of gold labeling of the alpha subunits of IL-2 receptor transiently expressed in HeLa cells. Note that imaging quality is decreasing with the increase in cell thickness.

FIG. 9j-k is a picture of an opened (i) and closed (j) automation-compatible elastic chamber.

FIG. 9l is a picture of individual parts of the automation-compatible elastic chamber.

FIG. 10a is a generalized axial cross-sectional diagram of the top part of housing of an automation-compatible elastic chamber.

FIG. 10b is a generalized longitudinal cross-sectional diagram of the top part of housing of an automation-compatible elastic chamber.

FIG. 11 is a generalized longitudinal cross-sectional diagram of the support part of an automation-compatible elastic chamber.

FIG. 12 is a generalized cross-sectional diagram of the plug of an automation-compatible elastic chamber.

FIG. 13 is a generalized cross-sectional diagram of the punch used for cutting the elastic film for the automation-compatible elastic chamber.

FIG. 14 is a generalized cross-sectional diagram of the stretching tool for stretching the elastic film for the automation-compatible elastic chamber.

FIG. 15a is a scheme of the preparation stage of a sample for imaging using the

automation-compatible oil chamber.

FIG. 15b is a scheme of the imaging stage of a sample using the automation-compatible oil chamber.

FIG. 16a shows a SEM image of the automation-compatible oil chamber from the top side.

FIG. 16b shows a SEM image of live HeLa cells viewed with the automation-compatible oil chamber.

FIG. 17 is a scheme of the automation-compatible inverted chamber.

FIG. 18 is an image of wet Live HeLa cells without any labeling or fixation obtained using the inverted chamber.

DETAILED DESCRIPTION OF THE INVENTION

Ideally, a technology for electron microscopic imaging wet samples, including but not limited to viable unfixed cells, should afford the following specifications: (i) High imaging properties, as well as high signal to noise and signal to background ratios; (ii) Physiological environment of the specimen i.e. conditions for in-vivo studies, specifically the ability to work in a wet environment with unfixed cell at minimal radiation damage thereby achieving minimal interference to imaging, and minimal perturbation to the biological relevance of the results; and (iii) High throughput platforms, that require minimal sample preparation steps and automation.

The current invention is based on chambers adapted for use with a Scanning Electron Microscope, which were designed to meet the specifications outlined above.

The present invention and the invention disclosed in co-pending International patent application PCT/IL01/01108 are based on a technology comprising the isolation of a fluid sample from the inherent vacuum required for operation of Scanning Electron Microscopes by the introduction of a membranous partition. The main advantages of this technology

are:

a. The sample can be maintained in liquid conditions, allowing work with intact cells.

b. It is possible to work at biologically relevant temperatures at or around 37°C.

c. There is no need for de-hydration or coating.

d. Fixation is an option, but not a requirement.

e. The cells are kept alive up to the beginning of the imaging. If radiation damage is minimized, there is the possibility to work with living cells.

f. Sample preparation is much less time-consuming and labor-intensive compared with standard EM techniques.

g. Consistent results are obtained with highly standardized preparation procedures.

h. There is a reduced likelihood of damaging the cell morphology and introducing artifacts during sample preparation. Thus, there is a greater opportunity to detect objects and phenomena that may not be detected with standard EM sample preparation protocols.

i. Compared with TEM techniques, this invention allows the survey of most of the cell surface and interior, rather than an arbitrarily restricted area obtained in thin tissue slices.

A membrane that is thin enough for energetic electrons to pass through and interact with the sample being studied encloses the wet environment provided in said chambers.

The partition membrane must have several important properties. First, it needs to be as transparent to electrons as possible. This implicates a low average atomic number (low Z) and a low density.

Specific mechanical properties are also required of the membrane. While keeping thickness as small as possible to minimize scattering of the electrons before they reach the

zone of interest, the membrane must resist a difference of pressure between the interior of the sample chamber and the surrounding vacuum for as large a surface area as may be required for observation. It must also be flexible enough to enable a considerable amount of handling in preparation of the sample, generally ruling out carbon films since they are very brittle. The porosity to the materials comprising the sample holder and inside the sample must be reduced as much as possible to ensure proper sealing of the chamber.

Ideally, the electrical conductivity of the partition membrane should be high enough to prevent the local charging of the external surface of the membrane, which may perturb the incident electron beam and blur the image. However, the aqueous environment of the sample may assist in removing local charging, allowing also poorly conductive membranes to be used.

Finally, the affinity of the partition membrane for the object observed may be an important factor. Typically in electron microscopes, the electrons go from the upper region to the bottom, so that the observed objects are located below the membrane. Thus, the best results are obtained when the objects are in close proximity to the membrane, and best when they are attached to it.

Different materials have been tested to build the membrane, all based on Carbon compounds. Formvar and Butvar, commonly used in TEM to build supporting films (Davison and Colquhoun, *J. Elec. Microsc. Tech.* 2:35, 1985; Handley and Olsen, *Ultramicroscopy* 4:479, 1979), and Polyimide have been tested. Of these materials tested to date only the last fully met all mechanical and sealing requirements. According to one currently preferred embodiment, polyimide membranes (e.g., Moxtex, Inc., Orem Utah, USA) were used in the current invention. Polyimide membranes show no measurable porosity to water and they can also resist the forces produced by atmospheric pressure in windows over 1 mm^2. To minimize this force on the membrane, and subsequent risk of

rupture, the surface was usually reduced by the use of a TEM Ni grid that was attached to the external side of the membrane (for example, see FIG. 16a). Affinity properties, if required, are achieved by internal surface treatments.

While many applications are possible using the invention described in the co-pending PCT application, entitled "Device and method for the examination of samples in a non-vacuum environment using a scanning electron microscope" (PCT/IL01/01108), it is difficult to utilize that invention for applications that require automation. With the setup disclosed in PCT/IL01/01108 the chamber is completely filled with water or an aqueous solution and hence the final closing requires delicate handling in order to avoid membrane rupture and is thus not well suited for large-scale automation. Another limitation that is formed in order to avoid membrane rupturing is the usage of a relatively thick membrane and hence the images produced are of limited resolution.

The current invention describes improved chambers, which provide a more practical technology platform for the performance and automation of wet sample imaging using SEM. Examples for applications of the current invention are disclosed. All chambers of the current invention are compatible with any SEM.

The improved chambers of the present invention comprise the following major advantages:

1. The chambers are suited for industrial automated manufacturing since they can be closed easily without rupturing the partition membrane. This improvement is achieved by filling only a small region inside the automation-compatible chambers with water or an aqueous solution while the rest contains air, which is simply compressed when closing the chamber.
2. The automation-compatible chambers are suited for disposable design since the screws can be replaced by simpler means to close the chambers, such as clip.

3. The automation-compatible elastic chamber and the automation-compatible oil chamber further allow usage of thinner partition membranes and consequently facilitate higher imaging resolution due to the reduced pressure inside the chamber while the sample is still maintained in a wet environment.

4. The inverted automation-compatible chamber disclosed here enables the observation of the surface of the sample which is accessible to the medium rather than the surface of the cells which is attached to the partition membrane. This is particularly advantageous for viewing labeled cells since most of the labeling is located on the surface of the plasma membrane accessible to the medium and may also be advantageous for viewing polar cell types wherein many regions of interest are located on the apical membrane side.

We now disclose in detail the mechanical and spatial setup and the properties required of the elements from which the automation-compatible chambers of the invention are comprised.

We disclose the characterization of the automation-compatible chambers, giving a guide to the choice of material and dimension. We further disclose the capabilities of the chambers in imaging a variety of different samples by means of non-limiting examples. The automation-compatible chambers may be adapted to numerous applications in diverse fields such as materials research and cell biology.

Description of the Preferred Embodiments

I. Automation-compatible chamber

Reference is now made to FIG. 1a, which is a generalized longitudinal cross-section diagram and pictures (FIGS. 1b-c) of the improved automation-compatible chamber. The chamber is composed of three main component parts: (a) Cavity disc [3] a disc (e.g., made

of Perspex) with a partition membrane [7] (e.g., made of Polyimide) and an optional grid (e.g., made of Nickel) glued to the outer surface of the partition membrane [7]. The cavity disc is where cells may be cultured and labeled (See FIG. 2a for a detailed drawing of this component and FIG. 2b for a picture); (b) Frame [2], an aluminum plate which is holding the cavity disc (See FIG. 3 for a detailed illustration and FIGs. 1b-c and 2b for pictures); (c) Housing, [1], the enclosing part of the chamber, made for example of Ertalyte (plastic), which is sealed to the frame and cavity disc by an O-ring [5] and screws [4] (See Fig. 4 for a detailed illustration and FIGs. 1b-c for pictures).

To prepare this improved chamber for viewing in the SEM, a cavity disc [3] is placed inside a frame [2] with the polyimide partition membrane facing downward, as shown in FIG. 2b. All handling steps from this stage are performed with the frame and the cavity disc together.

Once a sample or samples are attached to the cavity disc and are ready for viewing, an O-ring (part **5** in FIG. 1a and FIG. 1b) is placed inside the housing and the chamber is tightly closed with screws [4].

To check that samples are vacuum tight, they are placed inside a vacuum chamber for a few minutes (until a pressure < 10^{-2} mbar is achieved).

II. Multi-sample handling

Figures 5-8 show a first embodiment of an assembly used for SEM imaging of multiple samples, using the chambers of the invention.

Figures 5 and 6b show the base of a multi-sample holder. The cavity disc frame of the chambers of the invention [2], with the cavity disc [3] inside, is placed in the base of the multi sample holder (FIG. 5 and FIG. 6b). The multi-sample holder is then closed by its cover [6] (See Fig. 6a, c) and tightened by four nuts.

Following this stage, the samples can be handled inside the multiple sample holder.

Liquids can be inserted into the cavities with pipettes (See FIG. 7c) and can be drained with the multi drain (See Fig. 7a-b) connected to a pump. The use of the multi drain [10] provides a safe way to take out liquids from the cavity discs since it is adjusted so that the needles or tubes [9] of the multi drain are kept at a safe distance from the partition membrane.

Once the samples are ready for viewing, the four nuts on the cover of the multi sample holder [8] are released and the ejector [12] with protusions [11] (See Fig. 8) is used to remove the cover [6] by placing it on top of the cover and lifting the cover with it.

III. Automation-compatible elastic chamber

Reference is now made to FIG. 9a, j, k and l, showing a cross-sectional diagram (a) and pictures of the improved automation-compatible elastic chamber, also termed hereinafter, the elastic chamber. The elastic chamber is a chamber design that allows a reduced pressure inside the chamber. This is important when the partition membrane used is susceptible to rupturing and specifically advantageous in cases when thinner partition membranes are used. This latter case can facilitate higher imaging resolution since with thinner partition membrane fewer electrons are scattered by the partition membrane. The reduced pressure is achieved in this case by replacing one of the chamber walls by an elastic or distensible film. This elastic or distensible film can expand when the chamber is placed in a vacuum environment thus leading to an increase in the volume of the chamber and consequently to a decrease in the pressure difference on the partition membrane.

The distensible or elastic film that should be used for this purpose should be distensible or elastic enough to allow a considerable change in the interior volume of the chamber on one hand and strong enough not to rupture or leak under a large pressure difference. One film found suitable for this purpose (by way of non-limitative exemplification) was a laboratory film, tradenamed Parafilm® of American National Can

(Chicago, IL, USA 60631).

The elastic chamber is constructed from five main component parts: (a) Cavity disc, 3, the same as in the automation-compatible chamber (See FIG. 2 for a detailed illustration); (b) Frame, 2, the same as in the automation-compatible chamber described above (See FIG. 3 for a detailed illustration); (c) Housing, part **13** (See Fig. 10 for a detailed illustration); (d) Support, **14**. The housing (3) and the support (part **14**) elements hold in between them a thin elastic film (e) Plug, **15**, placed a the bottom part of the support element and also ensures that the elastic film does not overstretch (See Fig. 12 for a detailed illustration).

To prepare an elastic chamber for viewing in the SEM, the initial steps until the closing of the chamber are similar to those for the automation-compatible chamber described above. Prior to closing the chamber, an O-ring (5) is placed in the support element (14). A precut piece of elastic film (cut using the punch shown in FIG. 13) is then placed in the bottom part of housing and the housing is then sealed to the support element while holding between these two parts the elastic film. Sealing is accomplished using screws (4). Using a stretching tool [18] (FIG. 14) the elastic film is pushed through the hole at the juncture of the housing and the support [19]. This maximizes the volume expansion of the chamber.

For closing the chamber, an O-ring is placed inside the top part of the housing (part 5) and the chamber is closed with the three screws. Finally, the plug (15) is placed at the bottom of the support element (14).

To verify that samples are in vacuum tight, they are placed inside a vacuum chamber for a few minutes (until pressure < 10^{-2} mbar).

IV. Automation-compatible oil chamber

The automation-compatible oil chamber, also referred as the oil chamber, is a design

of the chamber in which the chamber is left open to the vacuum environment, yet the sample remains in a wet environment. A layer of low vapor pressure liquid separates between the vacuum environment of the electron microscope and the sample, which is maintained in a wet environment. The wet medium of the samples is also termed herein the water layer or the sample liquids.

The configuration of the oil chamber reduces the pressure on the partition membrane to pressures of the order of the water vapor pressure (17 Torr at room temperature) and simplifies handling considerably. The low pressure on the partition membrane reduces even further the possibility for partition membrane rupturing and allows using thinner partition membranes than in other chamber designs.

The basic idea behind the oil chamber is to use a layer of a suitable liquid as a separation layer between the aqueous layer of the sample and the vacuum. The separation layer, also termed herein the "oil layer", or in other words the liquid used as a separation layer should have the following properties:

(i) A low vapor pressure, in order that high vacuum can be achieved inside the vacuum chamber.

(ii) Immiscible with aqueous media to avoid mixing between the sample liquids and the separation layer.

(iii) High surface tension, so that after turning the cavity disc upside down the aqueous layer of the sample and the separating oil layer stay in their relative position, and the sample attached to the partition membrane, stays in a water environment.

(iv) A specific gravity smaller than that of water in order to allow the separation layer to float over the sample liquids. In principle, it is possible to turn the cavity disc after adding the sample aqueous layer and then to add from below a liquid heavier than water, however, this procedure complicates handling considerably.

The use of low vapor pressure oils for reducing evaporation of the liquid reagents from tissue sample viewed by laser capture microdissections, is disclosed in US Patent No. 6,157,446.

In a most preferred embodiment, the liquids used for the separation layer are, by way of a non-limiting example, low vapor pressure oil, paraffin oil or TKO-w/7 (Kurt J. Lesker Co., Pittsburgh PA, USA).

Reference is now made to FIG. 15, showing an illustration of the stages of preparation and imaging with the oil chamber. The sample is first attached, or grown in the case of cells, on a cavity disc in a similar manner to the way it is described in the inventions above (see FIG. 15a). Then, a layer of oil is applied on top of the aqueous layer of the sample. The cavity disc is then turned upside down (see FIG. 15b) and inserted into the microscope's vacuum chamber. In this way the oil prevents the evaporation of the aqueous layer inside the vacuum chamber. The relative position of the oil and aqueous layers stays the same after turning the cavity disc upside down due to surface tension.

In addition to the choice of liquid, the chamber design should be such that surface tension will prevent spilling of the liquids from the cavity disc as well as a change in the relative positions of the two liquids after turning the cavity disc upside down. This essentially implies a limitation on the volume inside the cavity disc.

It is also preferred to place the cavity disc in a vacuum chamber before turning it upside down in order to allow any trapped gas bubbles to emerge of the liquids since the presence of gas bubbles might cause the oil layer and water layer to exchange positions inside the microscope chamber.

V. Automation-compatible inverted chamber

The water environment inside the wet chamber limits the penetration depth of the

electron beam to a few microns. Thus, samples can be imaged effectively only at regions close to the partition membrane. This detail becomes limiting when observing labeled cells since most of the labeling is located on the far side of the plasma membrane – looking from the partition membrane side. Furthermore, in polar cells, many regions of interest are located on the apical membrane side, which is again on the far side of the plasma membrane. To solve these problems an inverted chamber was invented.

The idea behind the automation-compatible inverted chamber, also termed herein the inverted chamber, is to grow the sample (e.g. biological cells) on a separate substrate rather than on the partition membrane, and to push this substrate with the cells grown on top of it, toward the partition membrane.

There major considerations in the design of the substrate used in the inverted chamber are: (a) the substrate should be located in close proximity to the partition membrane in order to observe the sample attached to it, thus it should be pressed towards the partition membrane; (b) the substrate must not break or disrupt the partition membrane, and therefore should be smooth, having no sharp edges; (c) the substrate may be elastic in order to acquire the shape that the partition membrane acquires when positioned inside the sample chamber.

Reference is now made to FIG. 17 showing a scheme of the inverted chamber based on the automation-compatible chamber described above, where a substrate in the form of a thin plastic film (19) is pushed by a piston (20) held by a spring (21). The sample is first attached, or grown in the case of cells, on the substrate (19) that is then placed, facing the partition membrane (7). The piston with the spring is placed inside the housing (1) of the chamber and then when closing the chamber the piston moves forward the substrate toward the partition membrane. The piston is designed to allow for excess fluid to escape.

Fig 18 shows wet live HeLa cells without any labeling or fixation, as imaged using an inverted chamber according to the present invention.

The inverted chamber can be integrated into other types of wet SEM chambers, including the ones described here. There is an advantage of using a chamber with lower pressure inside since then the partition membrane is not distorted stretched or ruptured.

It is appreciated that various features of the invention which are, for clarity, described in the contexts of separate embodiments may also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment may also be provided separately or in any suitable subcombination.

The following examples of the principles of the present invention are provided solely for illustrative purposes intended to be construed in a non-limitative fashion.

EXAMPLES

Example 1 – SEM images of HeLa cells using the elastic chamber.

Figures 9b-h show images of cells acquired using the elastic chamber. Fig 9b demonstrate the high signal to noise ration (SNR) achieved using immuno-gold labeling. Labeling was applied using a transient expression of the alpha subunits of IL-2 receptor in HeLa cells. Both, transfected and non-transfected cells are shown in the same field, demonstrating the exquisite signal to noise ratio.

Figures 9c-h present a series of images at high magnifications of gold-labeled HeLa cells using an anti EGF receptor antibody.

Example 2 – SEM images of HeLa cells using the oil chamber.

A SEM image of the oil chamber, from its top, is shown in FIG. 16a. The darker gray circle in the middle of the images reflects the region filled with water. The brighter signal surrounding the water circle represents the glue that was used for the attachment of a plastic disc to the partition membrane. The white grid in the figure is the metallic support grid and the stripes around the perimeter of the water region are wrinkles in the partition membrane. Wrinkles in the partition membrane provide evidence for the relatively low pressure applied to the partition membrane in the set up of the oil chambers.

Figure 16b shows a magnified SEM image of HeLa cells, in vivo, attached to the partition membrane of an oil chamber.

Example 3 – Dependency of gold-labeling resolution on cell thickness.

The limited penetration depth of the electron beam in a water environment inside the wet chamber is demonstrated by Figure 9i. This figure shows HeLa cells transiently overexpressing IL-2 receptor, labeled with 40 nm gold particles. The labeling is clearly detected on the thinner parts of the cells, near the cell edges, but cannot be detected on the center parts of the cells, like the nucleus area. The labeling at the far side of the cells can be detected using the inverted chamber of the current invention.

Example 4 – Drug discovery applications of the current invention

The reliable and automation-compatible imaging of wet samples that is enabled by the devices of the current invention opens opportunities for applications in drug discovery. The invention particularly applies to a plurality of disease areas in which a relatively small number of membrane proteins (e.g. receptors) plays a role, as well as disease areas in

which the pattern or localization of receptors plays a role, such as viral infections (e.g. HIV-1 infection of CD4+ cells), genetic disease (e.g. cystic fibrosis), and others. The high sensitivity enables the study of viable non-fixed cells, a long felt need of life science.

A functional cell-based assay for selecting compounds that inhibit the entry of HIV-1 into its target cells is disclosed as an illustrative example. It is based on the knowledge that CCR5 and CD4 are the principal receptors used by HIV-1 for entry into cells in primary infection. Affecting CCR5 expression, spatial distribution, and/or co-clustering with CD4 is believed to be a potent anti-HIV-1 treatment strategy. The prior art does not disclose a system (device and method) with sufficient quality (resolution, throughput, biological relevance and so forth). Specifically, optical microscopes, of which resolution is limited by the wavelength of light, are practicality limited for resolution in the sub micron level. In contrast, the high resolution of Electron Microscopy (EM) can reveal in-depth biological information (Singer II, et al. 2001. *J Virol* 75:3779-90). However, conventional EMs are a cumbersome, since manual system cannot be used efficiently for drug discovery. So far, optical-fluorescence microscopy and Transmission Electron Microscopy (TEM) were used as preferred technologies for CCR5 and CD4 studies. Fluorescence microscopy is limited by resolution and thus reveals unreliable results when trying to detect patterning of membrane proteins (Singer II, et al. 2001. *J Virol* 75:3779-90), and TEM is a cumbersome tool to be employed for screening drug candidates. Gene transfection and over expression are required to overcome the low sensitivity of fluorescence-based techniques, and drastic procedures (Freeze-fracturing or embedding and sectioning) during sample preparation for TEM may bias the results and limit the ability to survey many regions on the cell.

The devices disclosed in the current invention enable imaging of biological samples, a detection scheme based on automated SEM. Similar schemes are already employed for quality assurance in the production of semiconductor devices. Such inspection tools are

disclosed in US Patents 6072178, 5644132, 5502306, 4618938, 4609809, and 4618938. Their use in biology is disclosed in a previous patent (PCT/IL01/00764).

The current invention allows quantifying protein levels in low-availability samples and/or low abundance proteins, where those are detected in the context of the cell. It enables the detection of known and new binding interactions of any of homo- and hetero-complexes. The current invention may be also utilized in diagnostics by performing protein profiling of small biopsies.

The invention may be used for the analysis of the cellular pattern of any plurality of biological molecules that may be of interest. This includes but not limited to: polysaccharides, small chemical molecules (e.g. lipids, peptides, hormones and other messengers, drugs) and any homo- (i.e. protein-protein as example) and hetero- (as protein with another protein, drug-protein, DNA-RNA, DNA-protein etc.) complexes as well as chemical modifications, whether or not naturally occurring.

Example 5 Detecting aggregation or oligomerization of molecules using a wet sample chamber

Many hits from high-throughput and virtual screening programs are subsequently found to have peculiar, undesirable characteristics as reviewed recently by Kirkpatrick (Nature Reviews Drug Discovery 1, 330, 2002). For example, they may act non-competitively, show little relationship between structure and activity, and have poor selectivity. Such hits can waste much time and effort, but despite their common occurrence, the underlying reasons for their behavior have remained unknown. Now, McGovern *et al.* (J. Med. Chem. 45, 1712-1722 (2002)) provide evidence that these compounds, although structurally unrelated, share the ability to aggregate, which results in them being falsely detected as hits in screening assays.

To account for the extreme sensitivity of the screening hits to the molar ratio of inhibitor to enzyme, the authors considered the hypothesis that the active inhibitor might be an aggregate of many molecules. Dynamic light-scattering experiments of aqueous solutions of the hits indicated the presence of particles with apparent diameters ranging from 95 to 400 nm these aggregates being much larger than the target enzymes, which are 20 nm at most in their longest dimension. The presence of aggregates was confirmed by McGovern and coworkers by transmission electron microscopy, and also backed up by further experiments on enzyme kinetics.

While light scattering, or TEM can measure aggregation a lot of work has to be devoted to each sample. The automation-compatible SEM chambers of the present invention have the advantage of parallelism and higher throughput.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which

carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same functions can be used; and it is intended that such expressions be given their broadest interpretation.

CLAIMS

1. A sample chamber for viewing wet samples with a Scanning Electron Microscope having at least one aperture sealable by a partition membrane, wherein said membrane separates the wet environment within the chamber from the vacuum of the electron microscope and wherein the membrane is adapted to withstand vacuum and is transparent to electrons, said chamber comprising a housing element enclosing a hollow space filled with a compressible gas.

2. The sample chamber according to claim 1, said chamber comprising:
   a. a cavity disc within the sample chamber having a top surface and a bottom surface wherein the top surface of the disc is sealed by the partition membrane;
   b. a frame element for holding the cavity disc, the frame element having an aperture extending at least over the width of the cavity;
   c. a housing surrounding the framed cavity disc having attachment means for attaching the frame to the housing, said housing enclosing a space filled with compressible gas.

3. The chamber according to claim 1 wherein the space filled with compressible gas can vary in volume.

4. The chamber of claim 3 wherein one wall of the sample chamber comprises an elastic or distensible element.

5. The elastic sample chamber of claim 4 comprising:
   a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane;
   a frame element for holding the cavity disc, the frame element having an aperture extending at least over the width of the cavity;
   a housing having an upper part and a lower part wherein the upper part of the housing extends from the frame on one end to an elastic film at the other end;

a support element placed below the housing and supporting it, having a top surface and a bottom surface surrounding a cavity in the support element through which the elastic film can be stretched, wherein the top of the support element extends to the housing and is covered with the elastic film, the bottom of the support element extends to a plug;

the plug placed at the bottom of the support element defining the maximum extent to which the elastic film can be stretched;

attachment means for attaching the frame to the housing and for attaching the housing to the support element.

6. The sample chamber of Claim 5, wherein said elastic or distensible film material is selected from the group of: elastomers based on polystyrene/elastomer block, copolymers, S-B-S, S-EB-S, S-I-S, Parafilm®.

7. A sample chamber for viewing wet samples with a Scanning Electron Microscope having at least one aperture sealable by a partition membrane, wherein the wet environment within said chamber is enclosed between said partition membrane and a separating liquid layer.

8. The sample chamber according to claim 7 wherein the wet environment within said chamber is enclosed between said partition membrane on one end and a separating liquid layer on the other end comprising:

a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane and the bottom surface is covered by a separating liquid oil layer;

optionally, a grid affixed to the outer surface of the partition membrane;

a frame element for holding the cavity disc, the frame element having an aperture extending at least over the width of the cavity.

9. The sample chamber of Claim 8 wherein separating liquid has at least one of the following characteristics: low vapor pressure, immiscibility with aqueous medium, high surface tension, specific gravity smaller than that of the solution that surrounds the sample.

10. The separating liquid of Claim 3, selected from the group comprising: low vapor oil, paraffin oil, TKO-w/7 oil, N-methyl pyrrolidone, gamma-butyrolactone.

11. An inverted chamber for viewing wet samples with a Scanning Electron Microscope having at least one aperture sealable by a partition membrane, wherein said membrane separates the wet environment within the chamber from the vacuum of the electron beam and wherein the membrane is adapted to withstand vacuum and is transparent to electrons, comprising:
a cavity disc having a top surface and a bottom surface wherein the top surface of the disc is sealed by a partition membrane;
optionally, a grid affixed to the outer surface of the partition membrane;
a frame element for holding the cavity disc, the frame element having an aperture extending at least over the width of the cavity;
a substrate having an upper side and a lower side, wherein the upper side faces the partition membrane; a substrate having an upper side and a lower side, wherein the upper side faces the partition membrane and a sample is situated on said upper side of said substrate;
means for positioning the substrate in close proximity to the partition membrane;
a housing element enclosing the framed cavity disc and positioning means;
attachment means for attaching the frame to the housing.

12. The chamber of any of Claims 1-11 adapted to hold aqueous medium.

13. The chamber of any one of Claims 1–11, wherein said membrane material is selected from the group comprising: polyimide, polyamide, polyamide-imide, polyethylene, polypyrrole and additional conducting polymers, parlodion, collodion, Kapton, FormVar, Vinylec, ButVar, Pioloform, silicon dioxide, silicon monoxide, carbon.

14. The chamber of any of Claims 1-11, comprising a wet sample mounted in proximity to the inner part of the partition membrane.

15. The chamber of Claim 14 wherein said wet sample comprises viable cells maintained under physiologic conditions.

16. The chamber of Claim 15 wherein the cells are cultured on the inner part of the partition membrane within the cavity disc.

17. The chamber of Claim 16 wherein the cells are cultured and labeled on the inner part of the partition membrane within the cavity disc.

18. The chamber of any of Claims 1-11, wherein said cavity disc is made of a plastic that maintains its shape and texture under the temperature and pressure that exist in said chambers.

19. A chamber holder adapted for holding a sample chamber according to any one of Claims 1-11.

20. The chamber holder of claim 19 adapted for holding an array of sample chambers.

21. The multiple chamber holder of Claim 20 enabling automatic exchange of samples during SEM examination.

22. The multiple chamber holder of Claim 20 enabling handling the samples within the multiple chambers placed in said holder.

23. Use of a chamber according to any one of claims 1-22 for screening drug candidates for a detectable effect on a cell component or marker.

24. Use of a chamber according to any one of claims 1-22 for detection of molecular interactions, aggregation or oligomerization.

For the applicants,
Cynthia Webb
Webb & Associates

ABSTRACT

The present invention provides chambers suitable for imaging a sample in a wet environment with a scanning electron microscope. The chambers comprise at least one aperture sealed with a membrane. The membrane is adapted to withstand a vacuum, and is transparent to electrons and the interior of the chamber is isolated from said vacuum. The chambers are useful for allowing multiple wet samples, including living cells, to be viewed under an electron microscope by an automated technology.

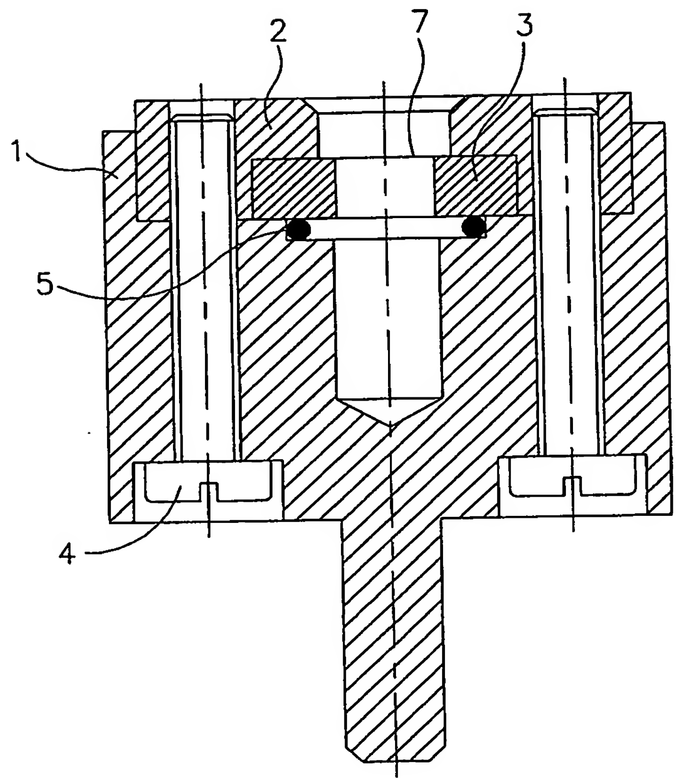


FIG.1A

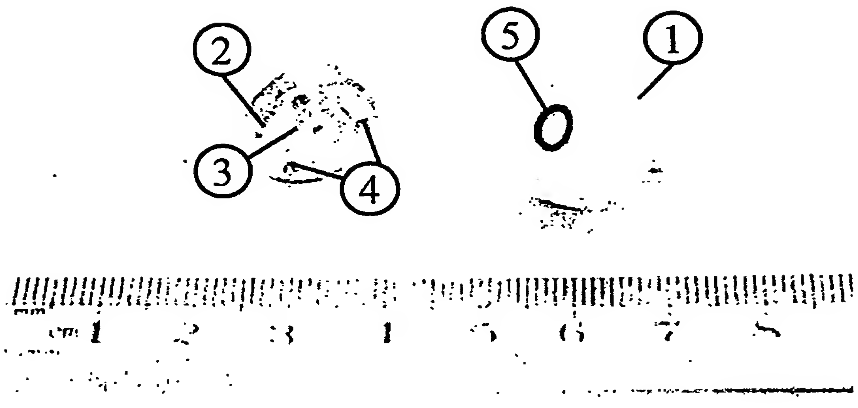


FIG. 1b

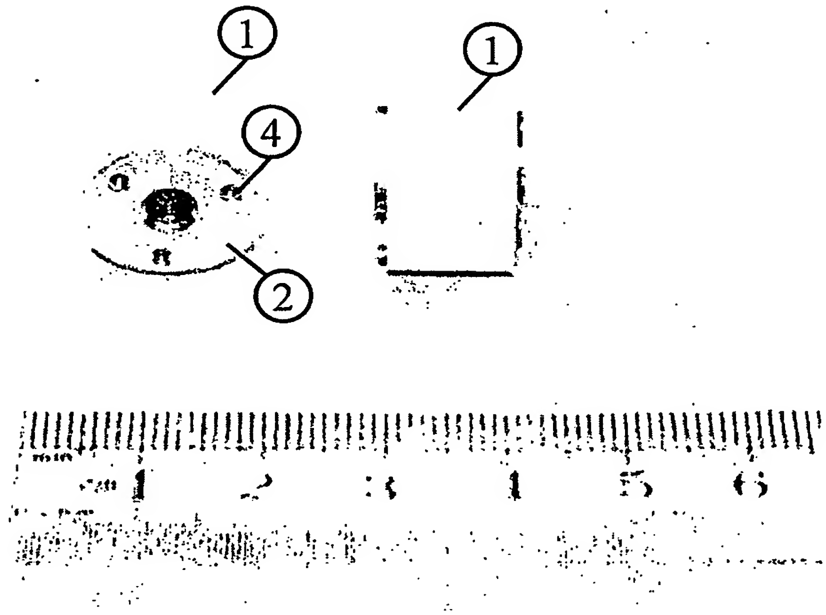


FIG. 1c

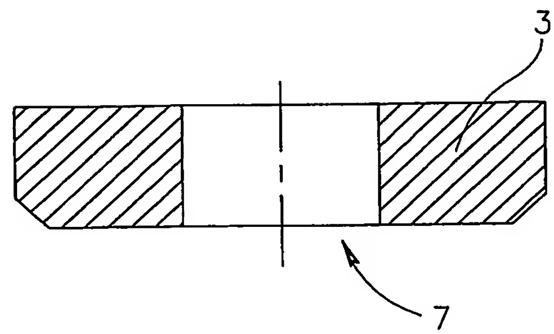


FIG.2A

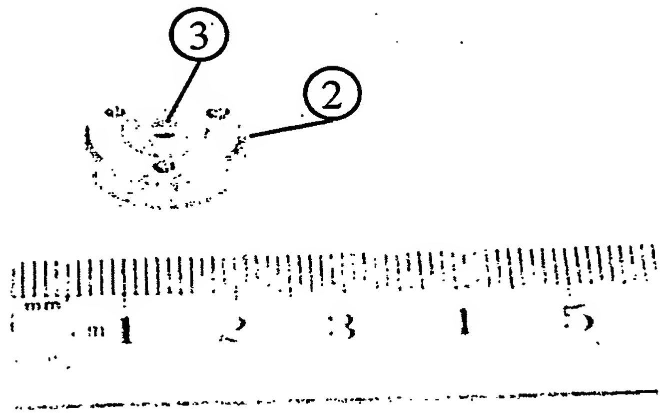


FIG. 2b

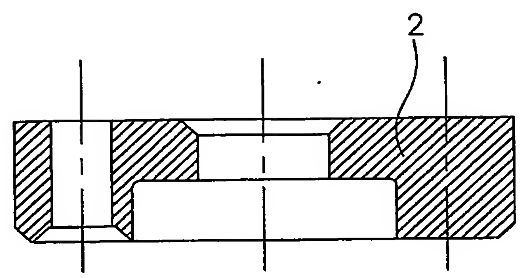


FIG.3

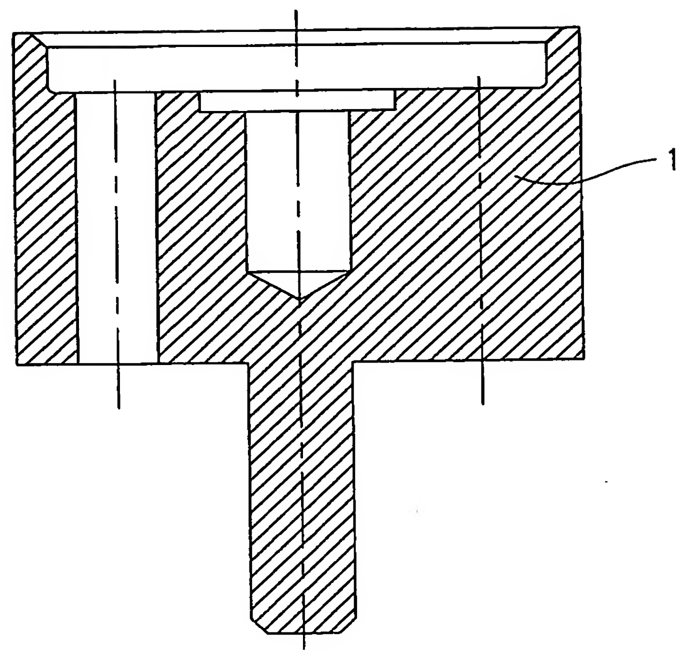
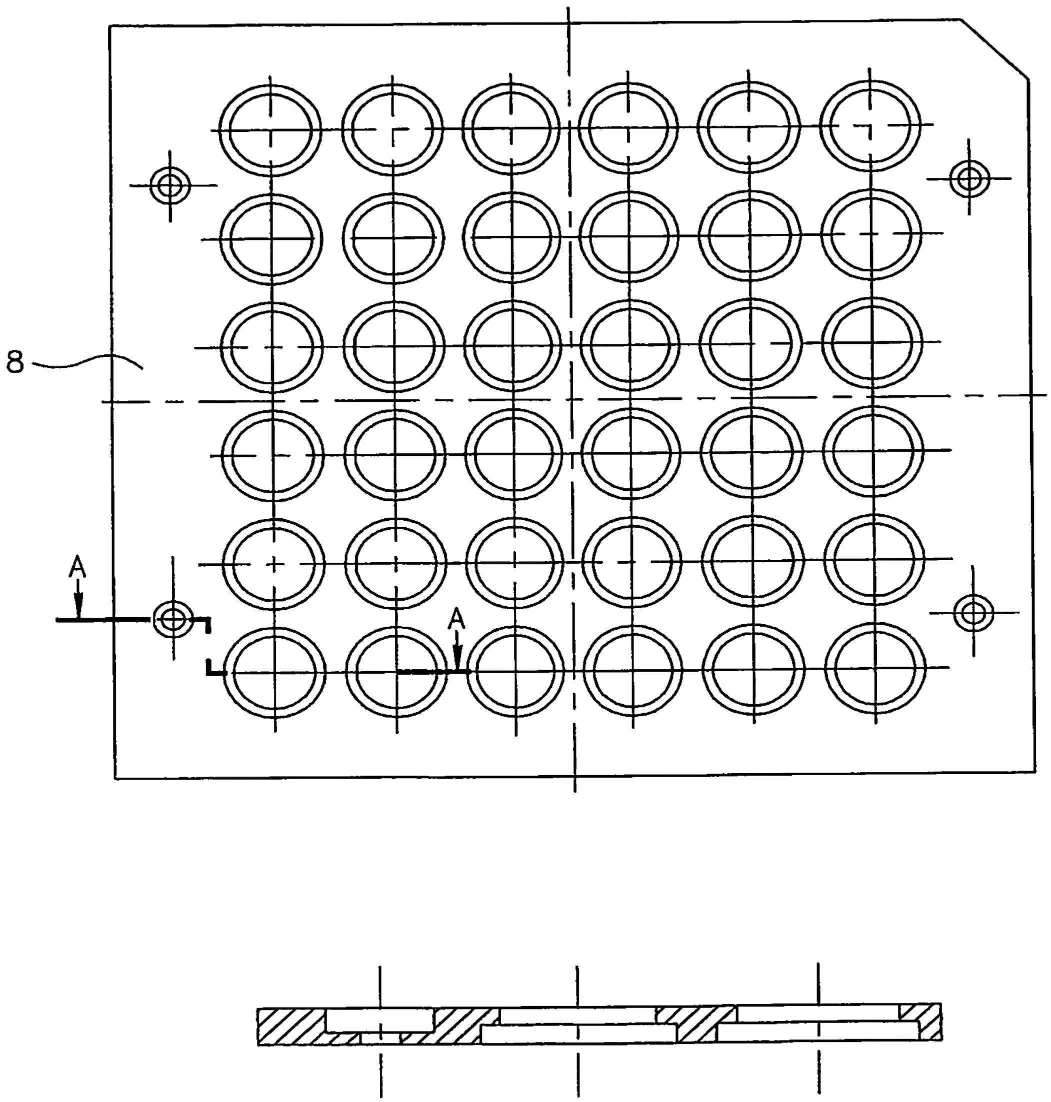


FIG.4



A–A

FIG.5

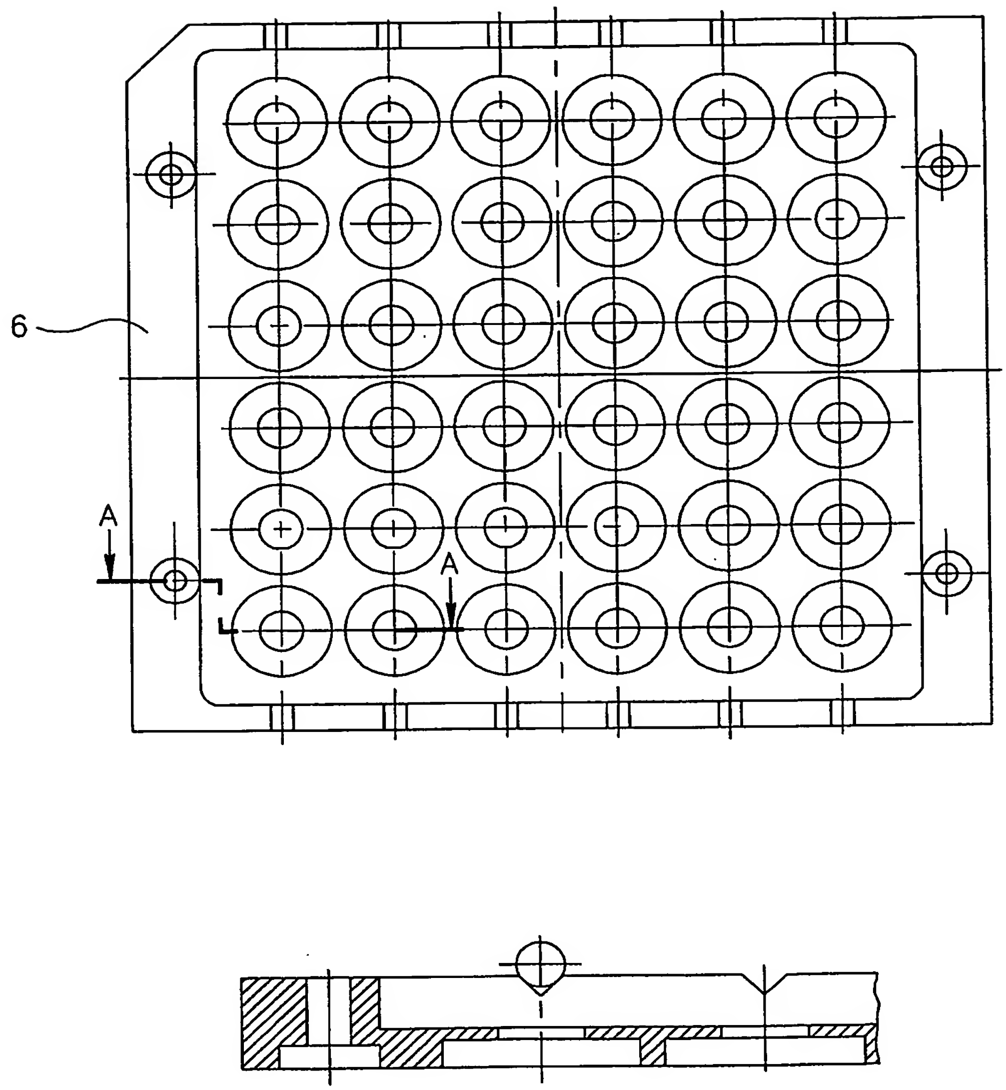


FIG.6A

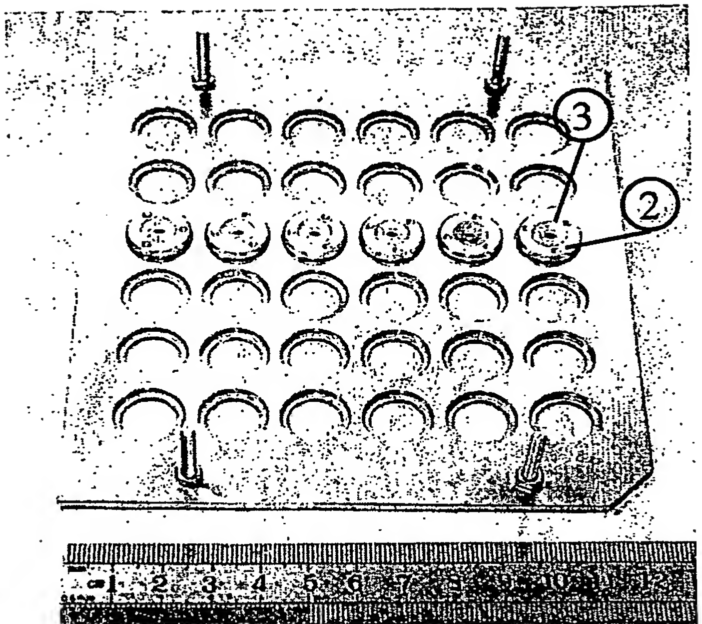


FIG. 6b

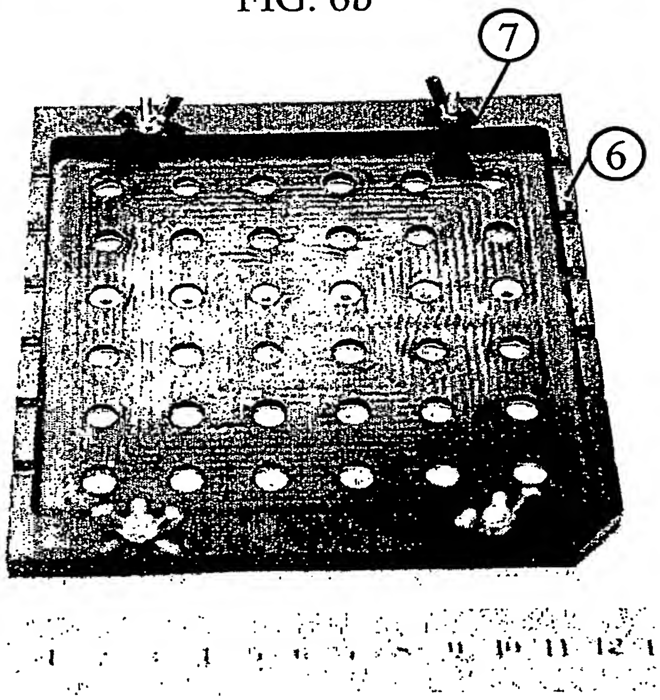


FIG. 6c

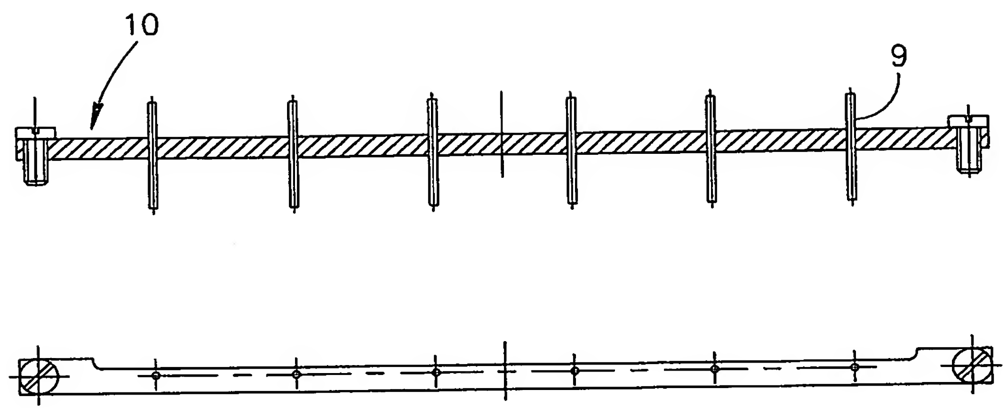


FIG.7A

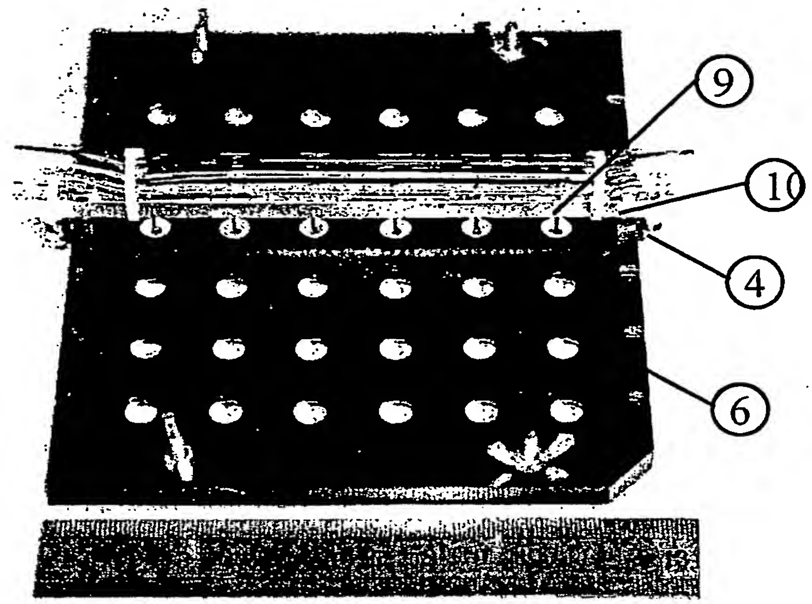


FIG. 7b

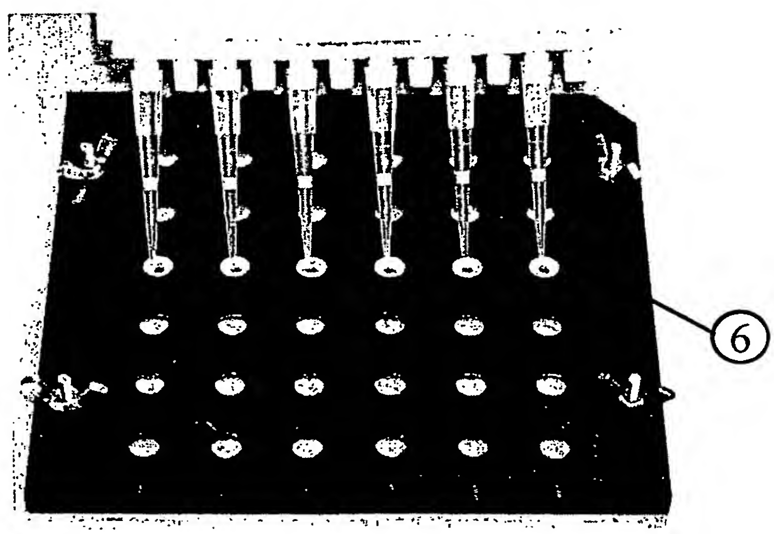


FIG. 7c

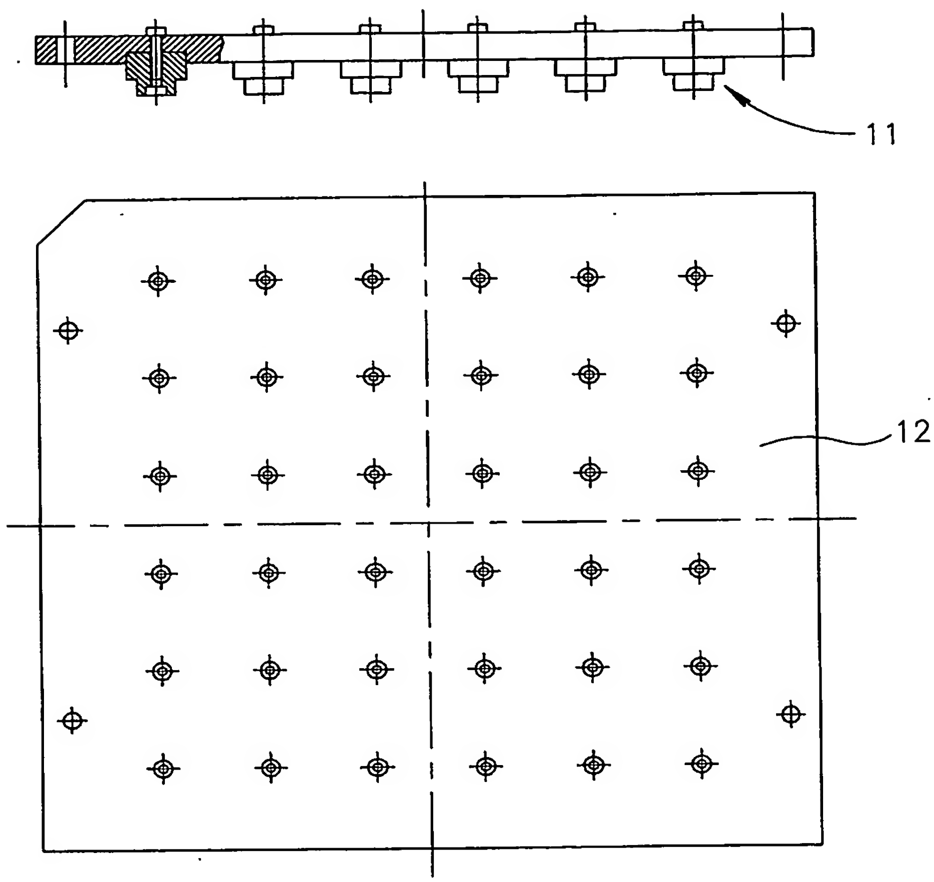


FIG.8

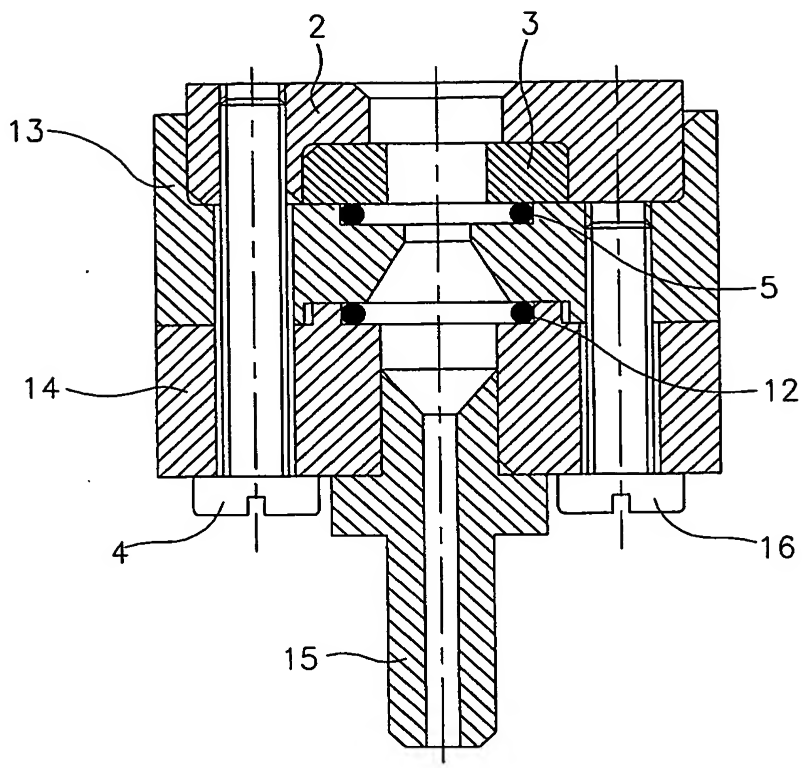


FIG.9A

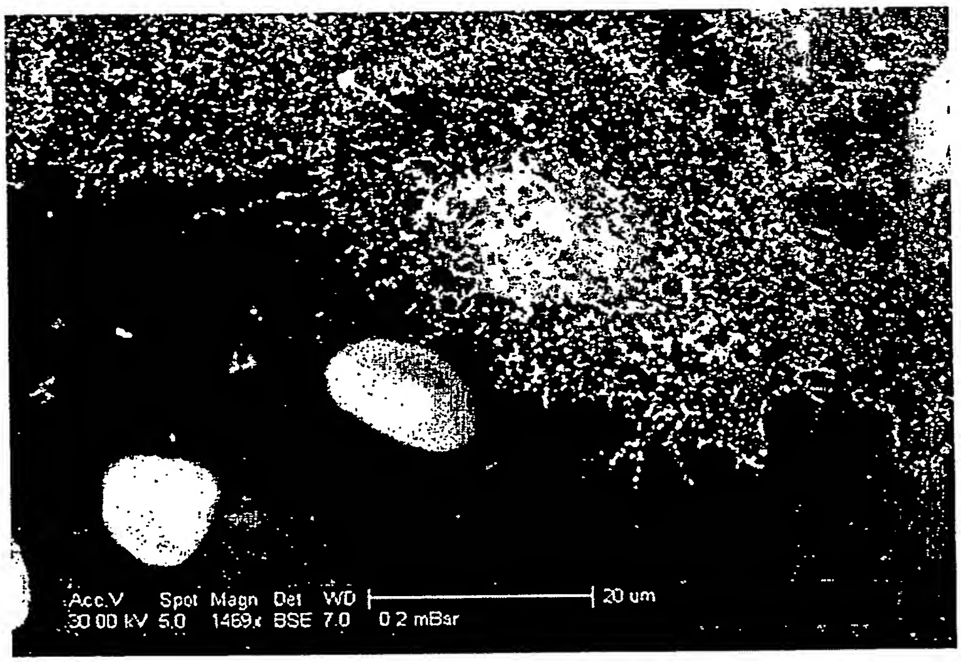


FIG. 9b

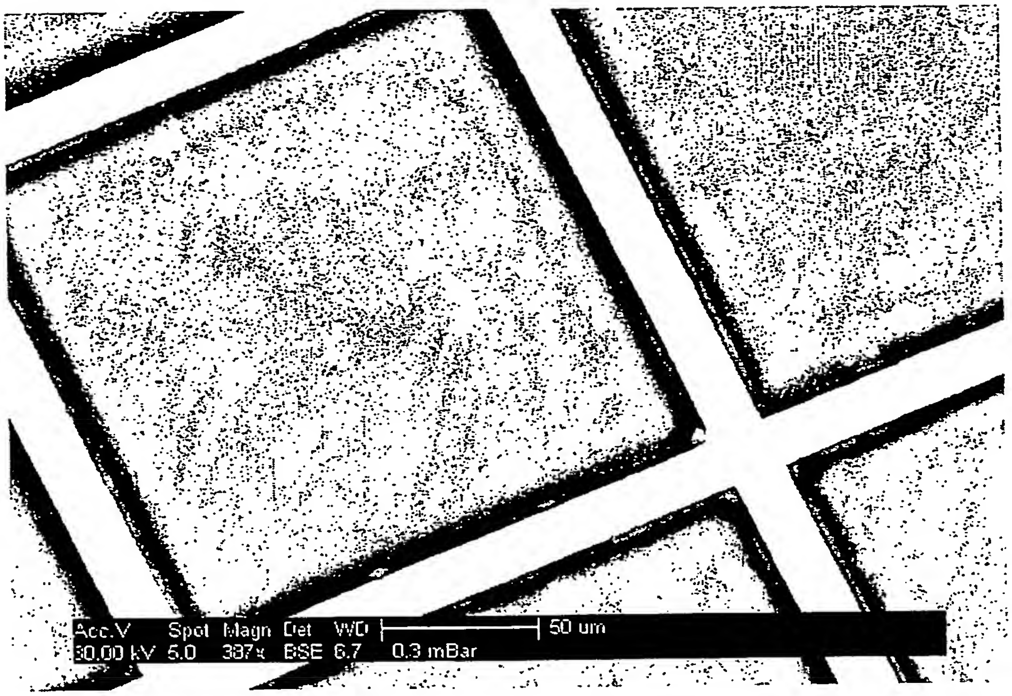

Acc.V Spot Magn Det WD 50 um
30.00 kV 5.0 387x BSE 6.7 0.3 mBar

FIG. 9c

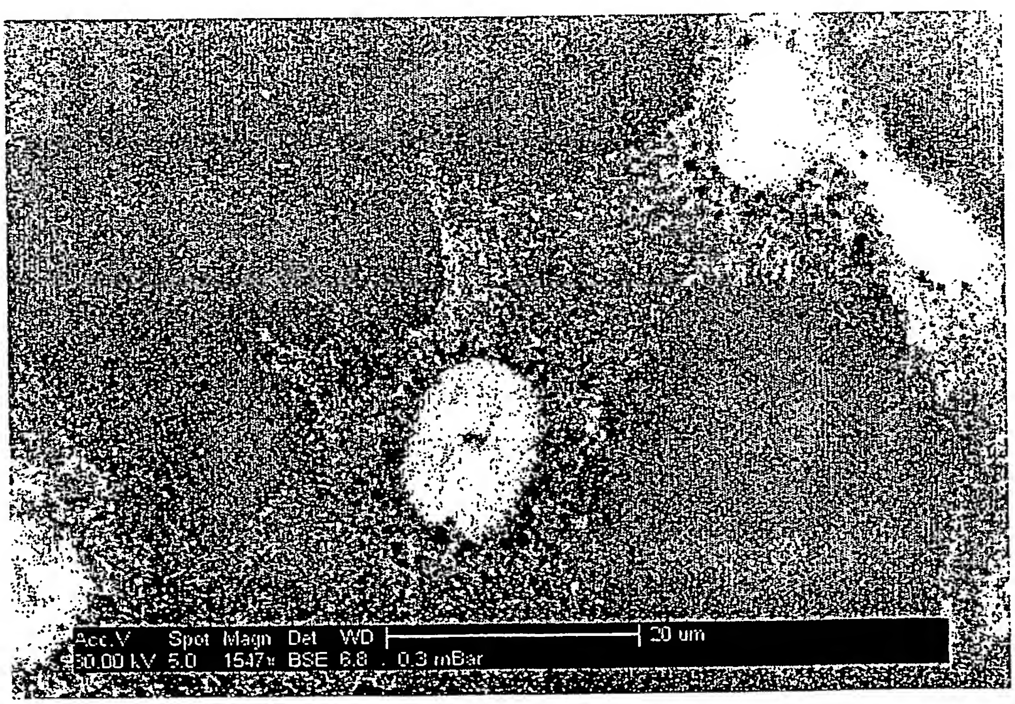

Acc.V Spot Magn Det WD 20 um
30.00 kV 5.0 1547x BSE 6.8 0.3 mBar

FIG. 9d

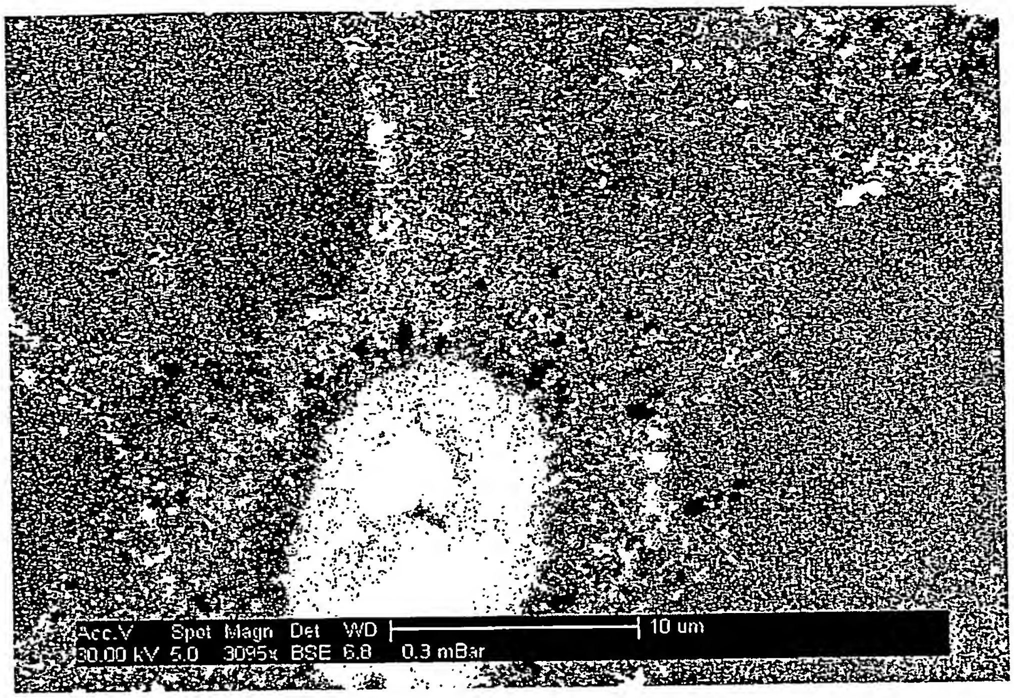

Acc.V Spot Magn Det WD 10 um
30.00 kV 5.0 3095x BSE 6.8 0.3 mBar

FIG. 9e

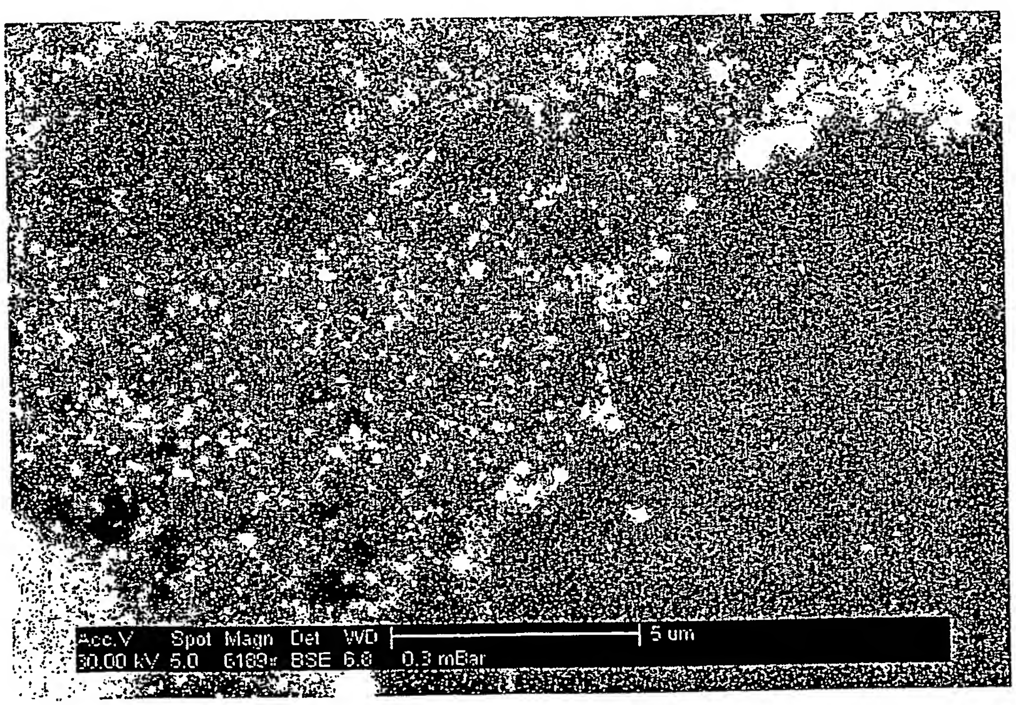

Acc.V Spot Magn Det WD 5 um
30.00 kV 5.0 6189x BSE 6.8 0.3 mBar

FIG. 9f

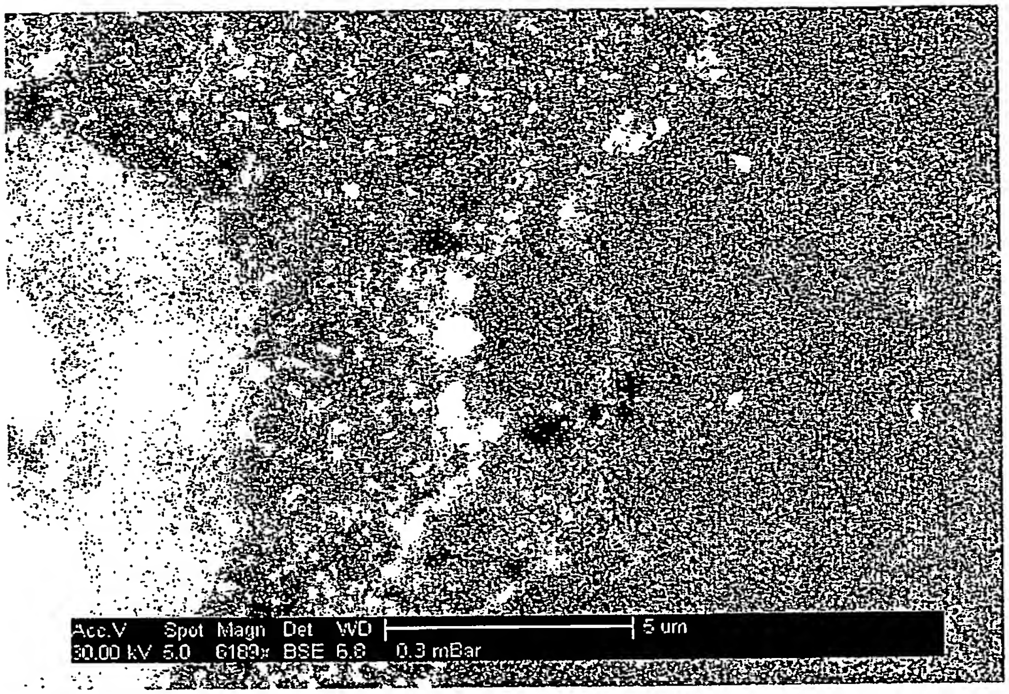

Acc.V Spot Magn Det WD
30.00 kV 5.0 6189x BSE 6.8 0.3 mBar
5 um

FIG. 9g

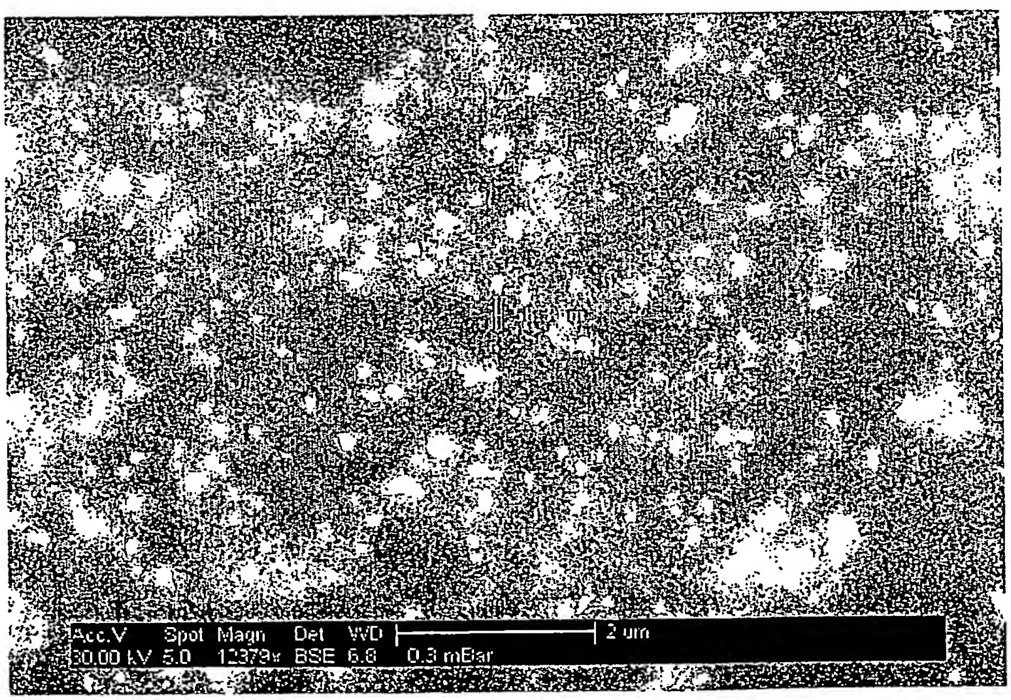

Acc.V Spot Magn Det WD
30.00 kV 5.0 12379x BSE 6.8 0.3 mBar
2 um

FIG. 9h

FIG. 9i

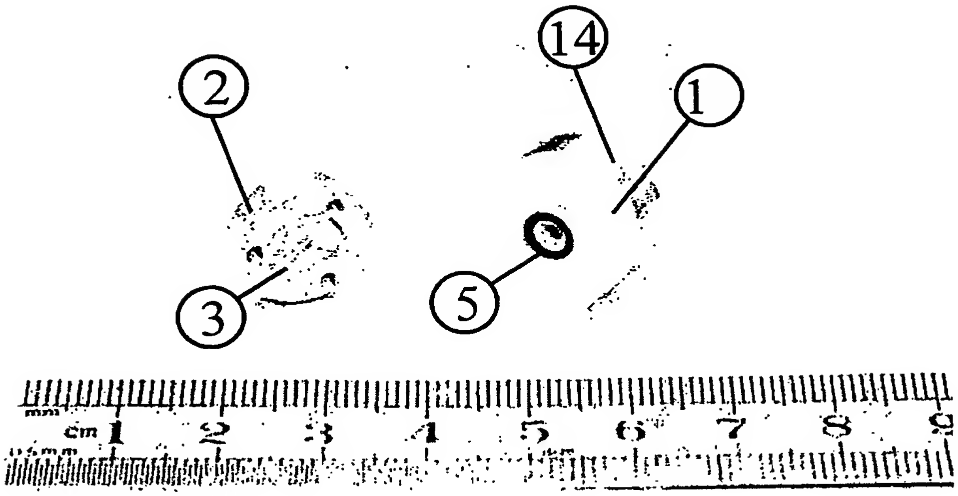


FIG. 9j

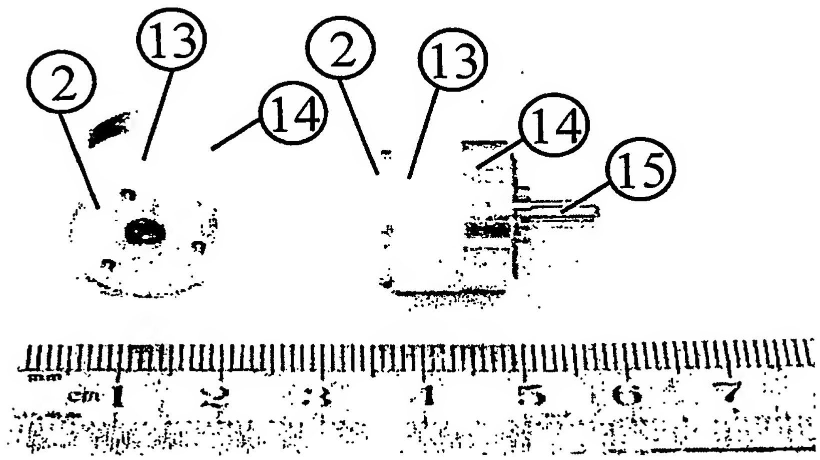


FIG. 9k

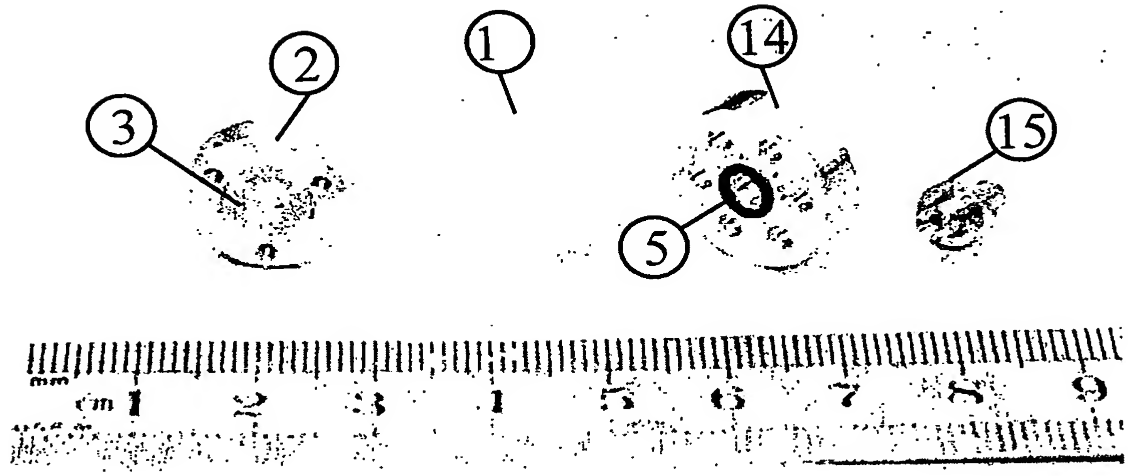


FIG. 91

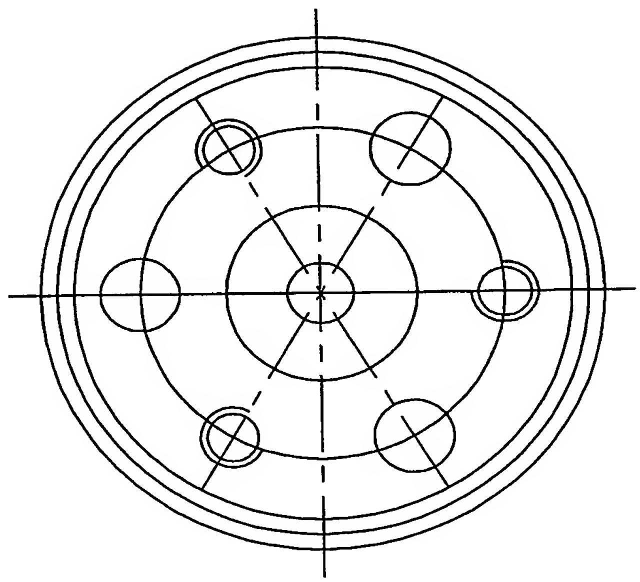


FIG.10A

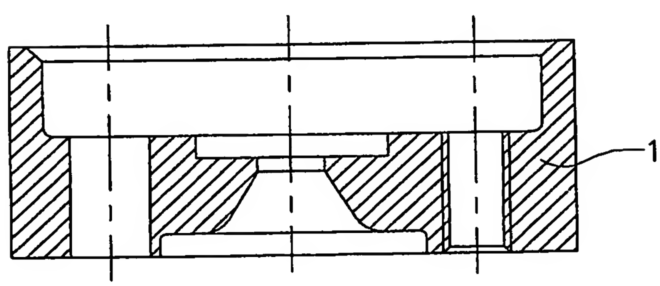


FIG.10B

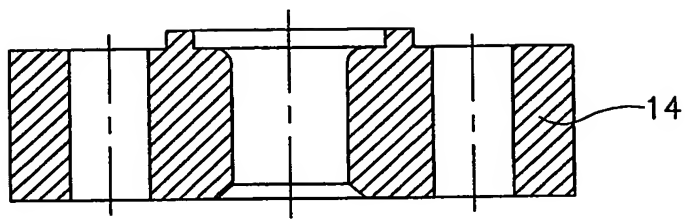


FIG.11

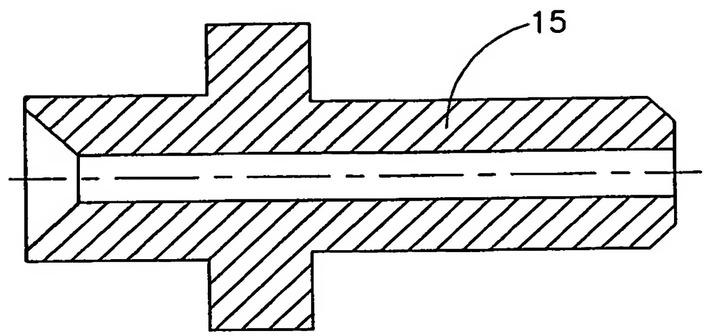


FIG.12

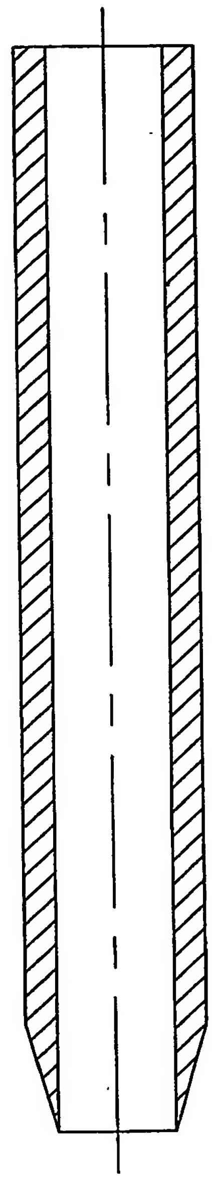


FIG.13

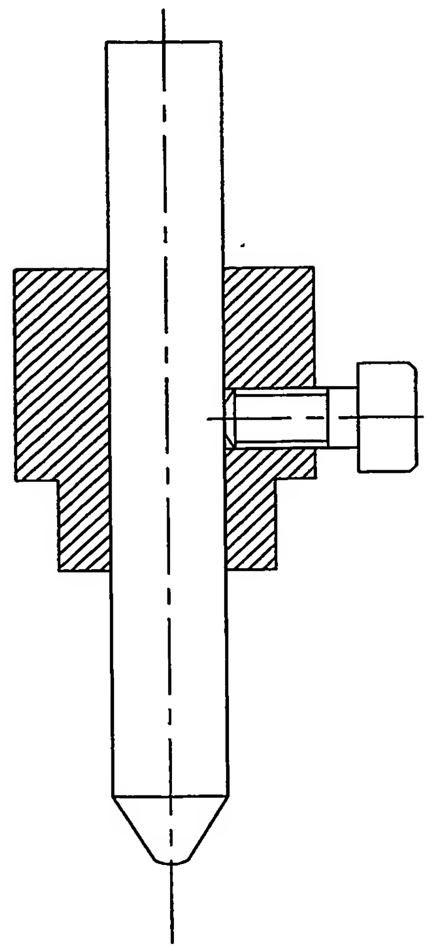


FIG.14

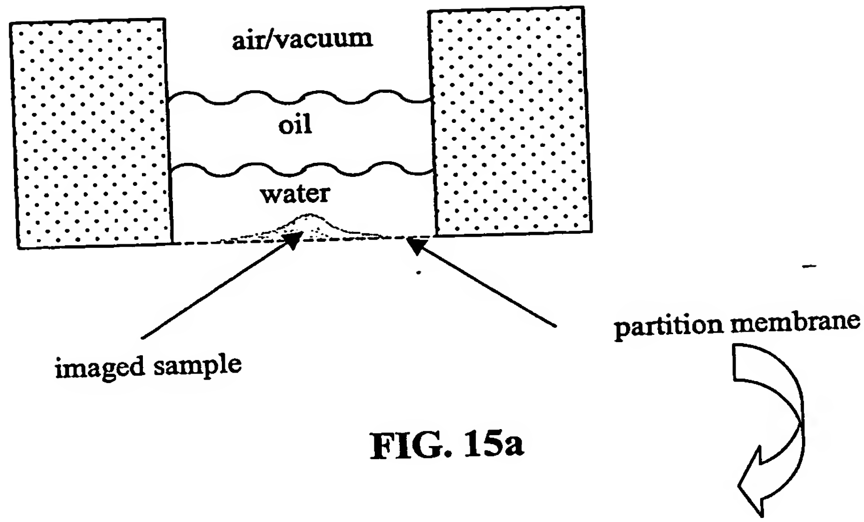


FIG. 15a

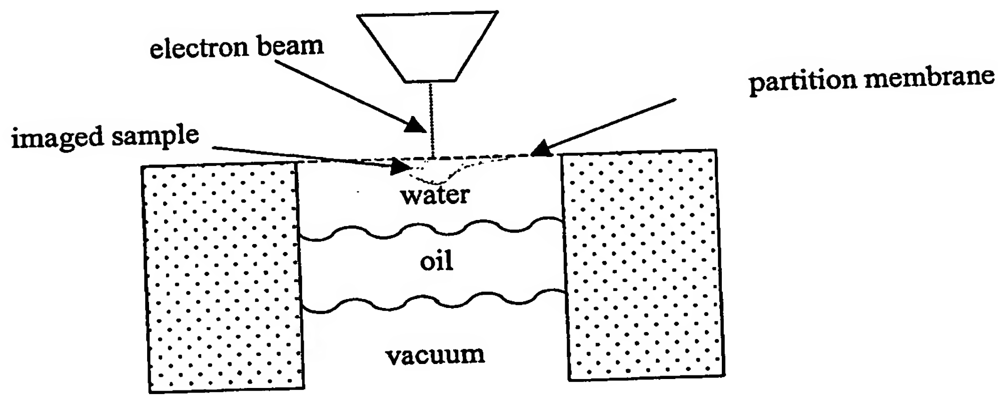


FIG. 15b

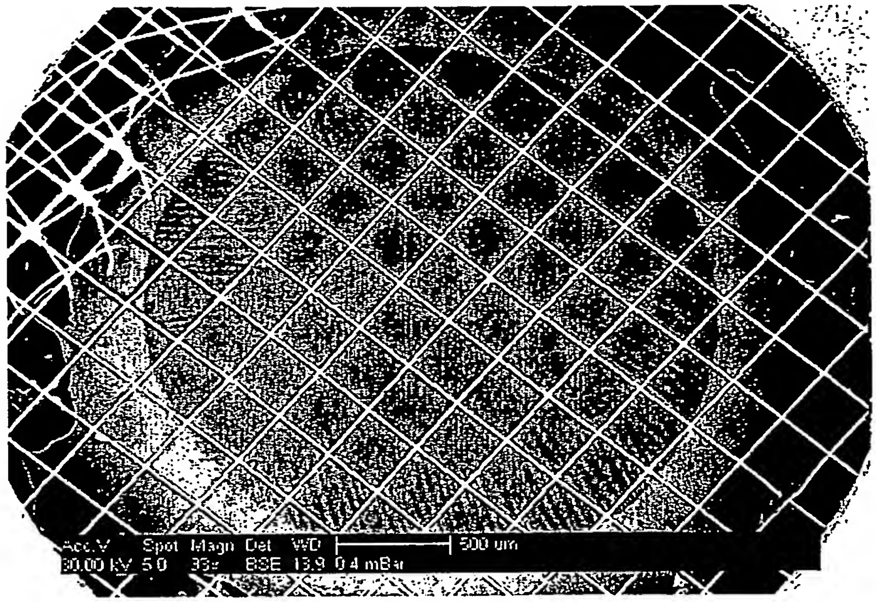


FIG. 16a

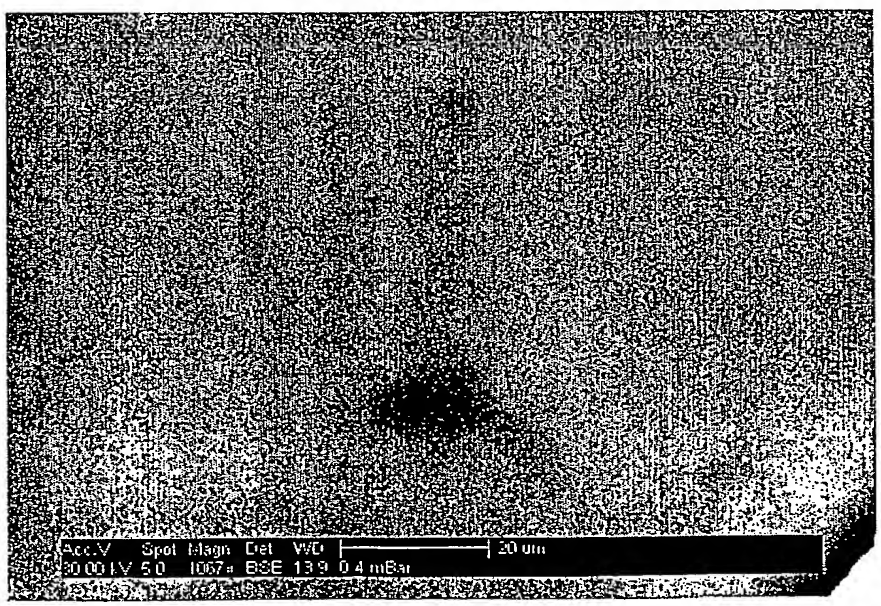


FIG. 16b

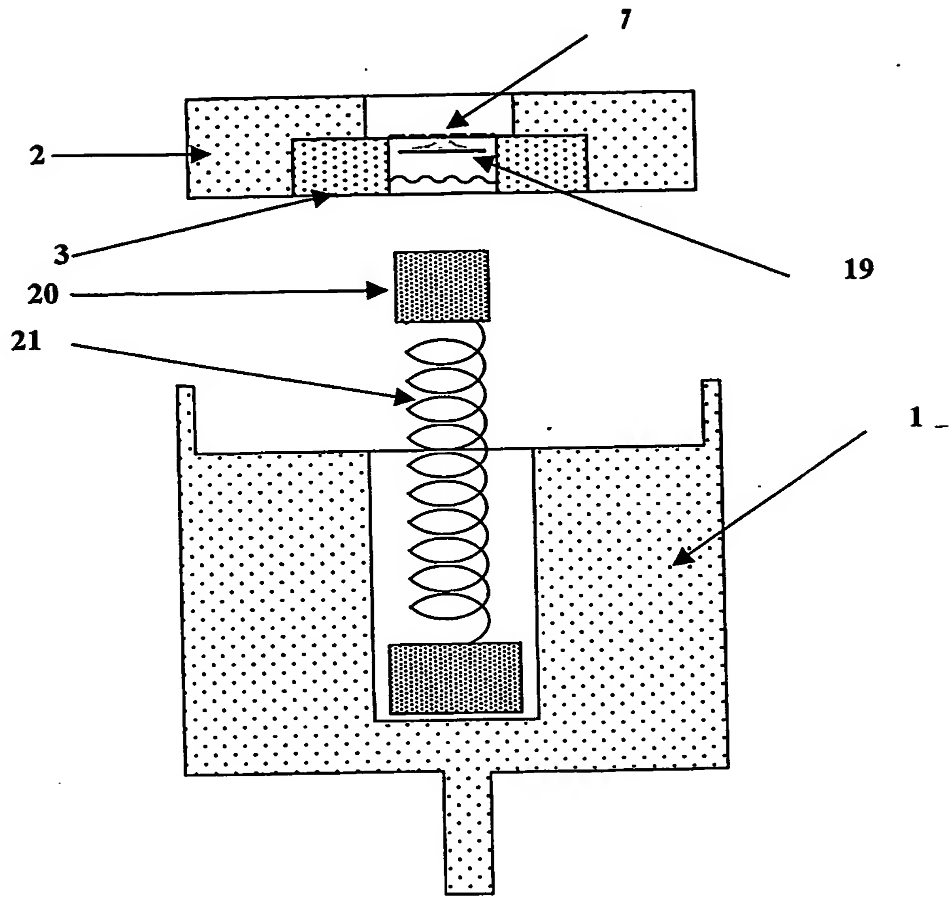


FIG. 17

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